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Research Paper

Competition of nuclear factor-erythroid 2 factors related transcription factor isoforms, Nrf1 and Nrf2, in antioxidant enzyme induction[☆]



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ABSTRACT

Although the Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) regulated expression of multiple antioxidant and cytoprotective genes through the electrophile responsive element (EpRE) is well established, interaction of Nrf2/EpRE with Nrf1, a closely-related transcription factor, is less well understood. Due to either proteolysis or alternative translation, Nrf1 has been found as proteins of varying size, p120, p95, and p65, which have been described as either activators of EpRE or competitive inhibitors of Nrf2. We investigated the effect of Nrf1 on EpRE-regulated gene expression using the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM) as models and explored the potential role of Nrf1 in altering their expression in aging and upon chronic exposure to airborne nano-sized particulate matter (nPM). Nrf1 knockout resulted in the increased expression of GCLC and GCLM in human bronchial epithelial (HBE1) cells. Overexpression Nrf2 in combination with either p120 or p65 diminished or failed to further increase the GCLC- and GCLM-EpRE luciferase activity. All known forms of Nrf1 protein, remained unchanged in the lungs of mice with age or in response to nPM. Our study shows that Nrf1 could inhibit EpRE activity in vitro, whereas the precise role of Nrf1 in vivo requires further investigations. We conclude that Nrf1 may not be directly responsible for the loss of Nrf2-dependent inducibility of antioxidant and cytoprotective genes observed in aged animals.

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Introduction

It has been well established that nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2) plays a key role in the regulation of many phase II detoxifying genes. Upon activation by electrophilic stimuli, Nrf2 is dissociated from Keap1 and then

translocated into the nucleus, where it binds to the electrophile responsive element (EpRE) located in promoters, thereby regulating the expression of specific genes, including many of the proteins categorized as Phase II detoxification enzymes. Studies have demonstrated that besides Nrf2, several other nuclear proteins also participate in the formation of Nrf2/EpRE complex and thus are involved in the regulation of these genes, including small Maf proteins [1], c-Jun [2], CBP [3], Bach1 [4], and c-Myc [5].

Another emerging mechanism of EpRE/Nrf2 regulation is mediated by a closely related Nrf family member, Nrf1. As summarized in Fig. 1, full-length Nrf1 (p120 Nrf1) binds to the endoplasmic reticulum (ER) membrane [6] and is apparently subjected to intramembrane proteolysis to generate nuclear, active Nrf1 (p95 Nrf1) [6–8]. In addition, there is a 23 kDa Nrf1 fragment that appears to be generated as a result of this proteolytic processing [9]. Another short form of Nrf1 (p65 Nrf1) is thought to arise from alternative translation from internal Met codons 321 and 326 that contain a stronger Kozak sequence, compared to Met1 [10]. The presence of the DNA-binding domains and the ability to bind to EpRE, while

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Abbreviations: EpRE, electrophile response element; ER, endoplasmic reticulum; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; GCLM, modifier subunit of GCL; HBE1, human bronchial epithelial cells; HO-1, heme oxygenase; Nrf1, nuclear factor-erythroid 2 p45 subunit-related factor 1; Nrf2, nuclear factor-erythroid 2 p45 subunit-related factor 2; nPM, nanoparticulate air pollution

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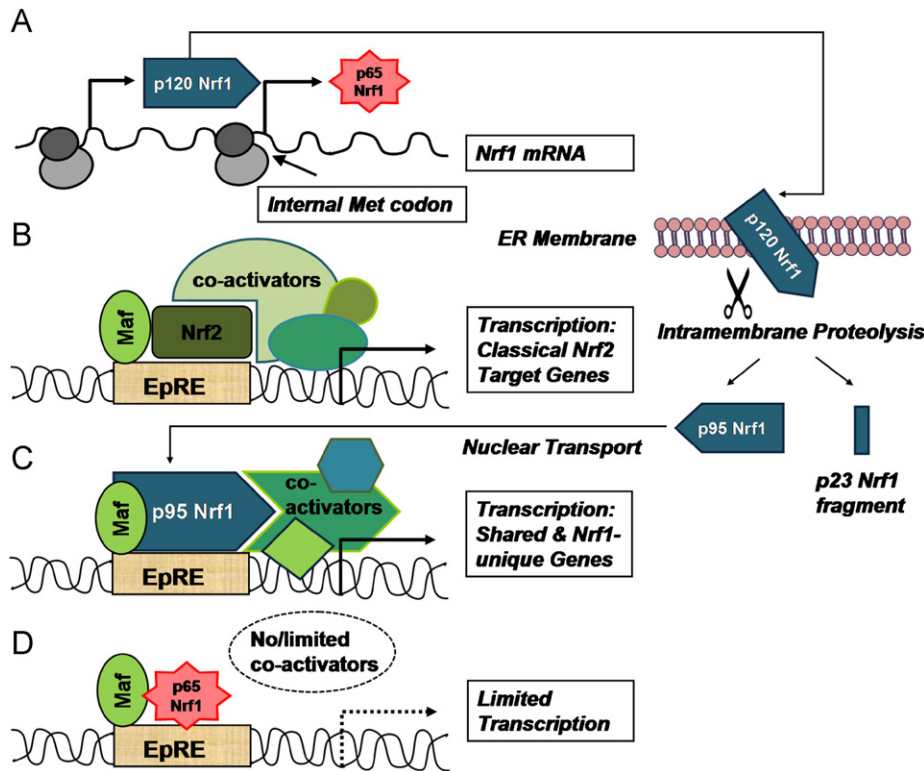


Fig. 1. General summary of competitive nature of Nrf1 forms towards Nrf2-mediated transcription. (A) Translation of entire Nrf1 mRNA coding region gives rise to a full-length Nrf1, migrating with an apparent molecular weight of 95 (non-glycosylated) or 120 kDa (glycosylated). Internal translation, from Met321 and Met326, that possess much stronger Kozak sequences compared to Met1, is thought to produce short form of Nrf1, migrating with an apparent MW of 65 kDa (p65 Nrf1, [10]). (B) In the absence of Nrf1, Nrf2 binds to EpRE and activates transcription of its target genes. (C) Full-length Nrf1 binds to the endoplasmic reticulum (ER) membrane, and possibly undergoes intramembrane proteolysis to generate nuclear p95 Nrf1 and p23 fragment. Once in the nucleus, Nrf1 competes with Nrf2 by recruiting a different set of co-activator proteins, controlling the transcription of Nrf1-unique set of genes [12]. (D) Short form of Nrf1 still binds to EpRE, but lacks certain transactivation domains and leads to diminished transcription of EpRE-controlled genes.

lacking one of the transactivation domains (AD1), allows p65 Nrf1 to act as a dominant negative inhibitor of the Nrf2/EpRE activity [11]. In line with that, the expression of Nrf2-target genes was increased in *Nrf2/Nrf1* double knock-out mice [12]. However, some studies demonstrated that Nrf1 is an enhancer instead of repressor of EpRE signaling [12], raising the concern of the exact role of Nrf1 in EpRE signaling and phase II gene regulation.

Based on the dissimilar co-activator domains in Nrf1 and Nrf2, it has been proposed that Nrf1 recruits a different set of EpRE binding proteins, resulting in Nrf1-unique expression of the EpRE-regulated genes [12]. Consistent with the inhibitory role of p65 Nrf1, it was demonstrated that hypoxia-mediated activation of overexpressed Nrf1 was accompanied by decreased expression of p65 Nrf1 while full-length Nrf1 was unaffected [9]. This supports the hypothesis that the abundance of p65 Nrf1 is regulated under different physiological conditions, presumably to control the extent of the responses to oxidative stress through the EpRE [11]. However, more studies are needed to further understand the function and regulation of p65 Nrf1.

In the current study, the role of Nrf1 in EpRE signaling and phase II gene regulation was explored using the glutamate cysteine ligase catalytic subunit (GCLC) gene as a model, as it has been shown to be regulated through EpRE/Nrf2 signaling [13]. Also the expression of Nrf1 in mice and its response to airborne nanoparticulate matter (nPM) was investigated as an extension of our previous finding that EpRE signaling and nPM-induced GCLC expression was impaired in middle-aged adult (21-month-old) compared to young (6-month-old) mice [14]. Evidence from the current study indicates that Nrf1 may work as an EpRE “repressor”, but its role in the regulation of Nrf2/EpRE signaling and the expression of phase II detoxifying enzymes remains to be explored.

Materials and methods

Reagents

Nrf1 siRNAs and antibody (sc-13031) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). M-PER, mammalian cell and NE-PER nuclear extraction reagents were purchased from Pierce (Thermo Fisher Scientific, Waltham, MA). Restriction enzymes and accompanying buffers were from New England Biolabs (Ipswich, MA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Luciferin was from BioShop Canada (Burlington, ON). The EMSA kit, including biotinylated EpRE probe from the human glutamate–cysteine ligase, modifier subunit (*gclm*) promoter was purchased from (Panomics, Fremont, CA). Lipofectamine™ RNAiMAX Transfection Reagent was from Life Technologies (Grand Island, NY). The complete protease inhibitor cocktail tablets and chlorophenol red-β-D-galactopyranoside (CPRG) were from Roche Diagnostics (Basel, Switzerland). Other basic chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids

N-terminally FLAG-tagged Nrf2 (pPROEX-HTC-Flag3-Nrf2) was obtained from Addgene (Addgene plasmid 21553 Cambridge, MA) and has been described previously [15]. Human Nrf1 cDNA was purchased from mammalian gene collection (accession number BC010623) and inserted into a modified pCR3.1 mammalian expression vector as previously described [9]. The V-5-tagged

p65Nrf1 was a kind gift of Dr. Jefferson Y. Chan from the University of California, Irvine and has been described previously [11]. The *GCLC*- and *GCLM*-EpRE-luciferase reporter plasmids (-3802*GCLC* 5'-luc and -1927*GCLM* 5'-luc) were a kind gift of Professor Dale A. Dickinson and their construction has been described elsewhere [16,17].

Quantitative analysis of mRNA

RNA from cells or homogenized animal tissues was extracted with TriZol Reagent. The total RNA was treated with DNA-free reagent to remove contaminating DNA. Then RNA was reverse transcribed and the mRNA contents of *GCLC* and *GCLM* were determined with real-time PCR assays using the protocol described before [18]. The primers were as following: *GCLC*, sense 5'-ATGGAGGTGCAATTAACAGAC-3', antisense 5'-ACTGCATTGCCACCTTTGCA-3'; *GCLM*, sense 5'-GCTGTATCAGTGGGCACAG-3', antisense 5'-CGCTTGAATGTCAGGAATGC-3'; GAPDH, sense 5'-TGGGTGTAACCATGAGAAG-3, antisense 5'-CCATCACGACA-CAGTTCC-3.

Cell culture, transient transfection and luciferase reporter assays

HBE1, an immortalized human bronchial epithelial cell line, was a gift of Dr. James Yankaskas at the University of North Carolina and were cultured as previously described [19]. Human embryonic kidney (HEK293A) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM supplemented with 10% horse serum and 3% P/S/A (300 units/mL penicillin G, sodium salt, 300 µg/mL streptomycin sulfate and 0.75 µg/mL Fungizone® in 0.85% saline (P/S/A)) in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were plated in 6-well plates at the density of 100,000 cells/mL and approximately 48 h later, upon becoming at least 70% confluent, were transiently transfected using Lipofectamine 2000, following the manufacturer's protocol. For each well of a 6-well plate, 0.2 ng of β-galactosidase, 3.0 µg of *GCLC* EpRE- or *GCLM* EpRE-luciferase and 1.0 µg of pCR3.1 (empty vector), Nrf1-FLAG, or p65Nrf1-V5 in combination with 1.5 or 2.0 µg of FLAG-Nrf2 myc-Nrf2 were used. Forty-eight hours later, cells were harvested, lysed and lysates were subjected to luciferase and β-galactosidase assays as previously described [9].

Gene silencing

Silencing of Nrf1 gene was performed with transient transfection of siRNAs. Briefly 50 nM of scrambled control or Nrf1 siRNA was transfected into HBE1 cells at 75% confluence, following the manual of the siRNA transfection kit (Life Technology). The cells were collected for assays after 48 h.

Airborne particle collection, mouse exposure and tissue processing

Airborne vehicular traffic particle collection and mouse treatment have been described previously [14,20]. Briefly, nanoparticles were collected with a High-Volume Ultrafine Particle Sampler [21] at 400 L/min in Los Angeles City near the CA-110 Freeway. This is considered to be the mix of fresh ambient particles mostly from vehicular traffic nearby this freeway [22]. The mean diameter of particles was 60 nm [20]. C57BL/6 male mice (3 month- and 18 month-old) were treated with re-aerosolized nanoparticles or ambient air (control) in sealed exposure chambers for 5 h/day, 3 days/week, for 10 weeks. Mice had normal weight and no signs of respiratory distress. After isoflurane anesthesia, mice were euthanized and the tissue was collected and stored at -80 °C until processing. For protein extraction, lung

tissue was ground in glass homogenizer using 100 µL of ice-cold CER I reagent from NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Rockford, IL), supplemented with protease and phosphatase inhibitors to prepare total protein lysate, according to the manufacturer's protocol. Protein determination was carried out using Bradford assay (BioRad, Hercules, CA).

Electrophoretic mobility shift assay (EMSA)

For EMSAs, 10–12 µg of protein lysate per reaction was used following the manufacturer's protocol (Panomics/Affymetrix, Santa Clara, CA). Briefly, after adding the labeled probe to the nuclear extracts, the mixtures were incubated at 15 °C for 45 min and subjected to the non-denaturing gel electrophoresis for 75 min at 120 V. For immunodepletion experiments, 2 µg of pre-immune serum (immunoglobulin G, IgG), anti-Nrf1, -Nrf2 or -Bach1 (sc-13031, sc-13031, and sc-21755) were added to the reactions and incubated for 2 h at room temperature as previously described [23].

Immunoblotting

For Western Blotting assay, 10–12.5 µg of protein per sample was run on the 10% or 12% SDS-PAGE for 1.5 h at 120 V. The protein was then transferred onto the Immobilon PVDF membrane (Millipore, Bedford, MA) at 180 mA overnight. The membranes were then probed with 5% milk in Tris-Buffered Saline, Tween-20 (TBST) for 1 h, followed by 1-hour incubations with primary and secondary antibodies with six five-minute washes in between. V5- tagged p65 Nrf1 was visualized using 1:1,000 dilution of V5 probe (sc-81594), followed by 1:4000 dilution of (goat anti-rabbit, sc-2004, Santa Cruz). Enhanced chemiluminescence substrate (Millipore, Bedford, MA) and Kodak X-Omat blue film (Perkin-Elmer, Waltham, MA) were used to achieve band visualization. Developed film was scanned using a CanoScan LIDE 80 scanner (Canon, Lake Success, NY) and AlphaEaseFC software, version 3.1.2 (Alpha Innotech/Cell Biosciences, Santa Clara, CA) was used for band densitometry.

Statistical analyses

In vitro data were analyzed using Student's t-test. Semi-quantitative data from mouse samples were analyzed using non-parametric Mann-Whitney Rank Sum Test calculator, available at: <http://www.holah.karoo.net/Mann-Whitney%20U-test.xls> and the results were confirmed with SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA, USA).

Results

Silencing Nrf1 induces *GCLC* and *GCLM* expression

Expression of *GCLC* and *GCLM*, both of which are regulated through Nrf2-EpRE pathway in human bronchial epithelial (HBE1) cells [19] was used to investigate potential contribution of Nrf1 to regulation of these genes. The effect of Nrf1 silencing (RNAi) was examined using real-time quantitative PCR. Nrf1 silencing resulted in approximately 1.7-fold increase in the *GCLC* and *GCLM* mRNA expression (Fig. 2A). The effectiveness of RNAi silencing was verified with Nrf1 immunoblotting (Fig. 2B). In addition to the forms of Nrf1 discussed here that migrated with an apparent molecular weight of 120, 95 and 23 kDa, in Fig. 2B, we note a band at approximately 30 kDa, in accord with what has been described regarding some other short Nrf1 forms elsewhere [24].

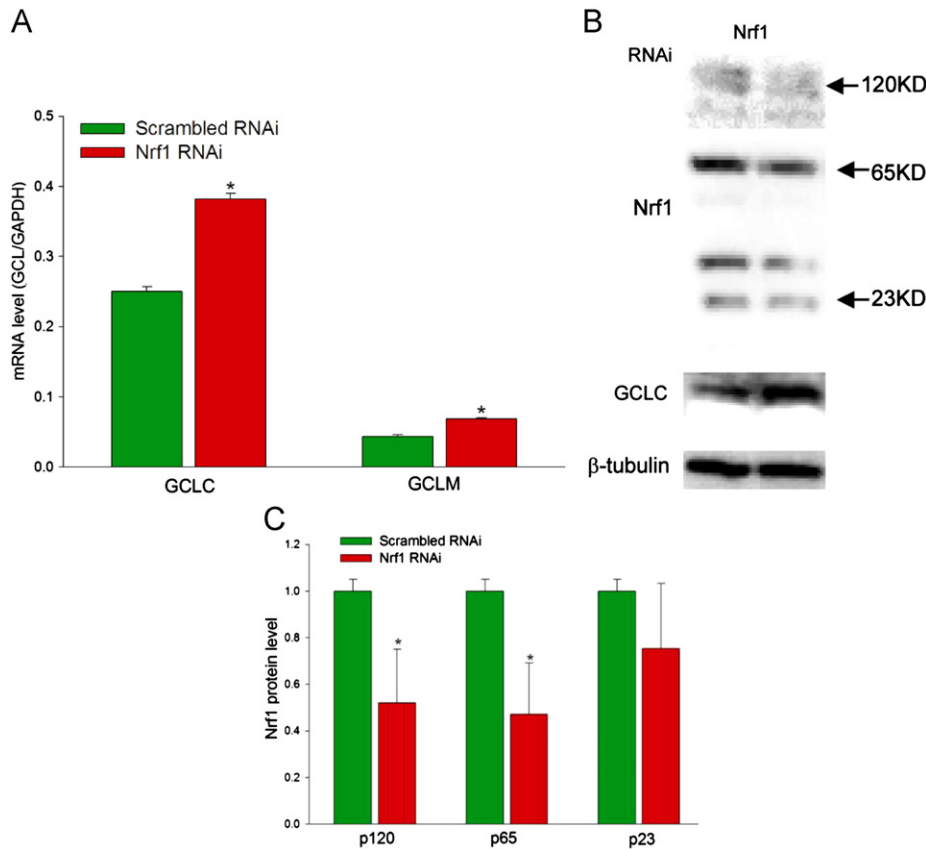


Fig. 2. Nrf1 silencing increased the expression of GCL genes. HBE1 cells were transfected with scramble or Nrf1 RNAi (final concentration 50 nM) for 48 h and collected for assays. (A) mRNA content of GCLC and GCLM in cells transfected with scramble RNAi (c) or Nrf1 RNAi (Nrf1); (B) Western blotting of Nrf1 and GCLC proteins in cells with/without Nrf1 silencing. $N=3$, $*$, $p < 0.05$ compared with control RNAi (c) (Student's t-test).

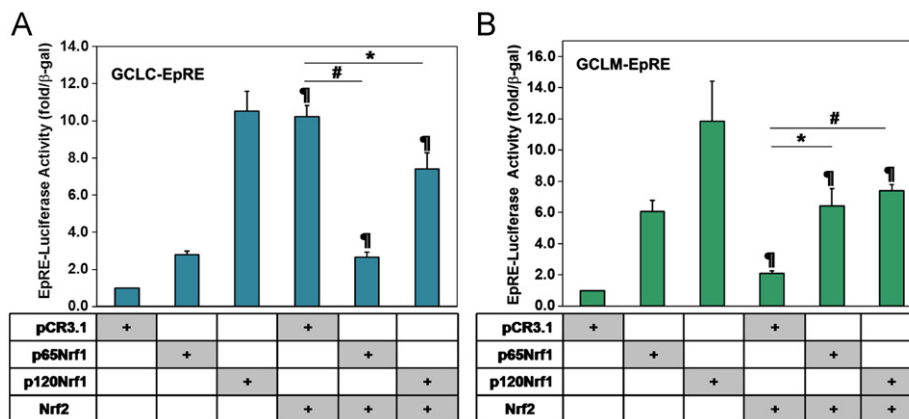


Fig. 3. Simultaneous overexpression of Nrf1 and Nrf2 diminishes or fails to further increase EpRE-driven luciferase activity. HEK293 cells were transfected with empty vector (pCR3.1), p65Nrf1, p120Nrf1 and Nrf2 along with the luciferase reporter vector with the EpRE from GCLC (A) or GCLM (B). β -galactosidase plasmid was used as the transfection efficiency control and the luciferase signal was normalized to β -galactosidase activity. Shown are means \pm S.E.M. of at least three independent experiments ($n \geq 3$). Asterisks ($*$) and the number signs ($\#$) indicate statistically significant differences in luciferase activity from lysates of cells, co-transfected with Nrf2 and empty vector pCR3.1 vs. Nrf2 and either p65 or p120 Nrf1 with $p < 0.05$ and $p < 0.001$, respectively (Student's t-test). Statistically significant difference ($p < 0.05$, Student's t-test) between mock (pCR3.1) and Nrf2-transfected samples is indicated by.

Simultaneous overexpression of Nrf1 and Nrf2 diminishes or fails to further increase EpRE-driven luciferase activity

We next investigated the involvement of Nrf1 in the regulation of Nrf2/EpRE activity using EpRE-driven luciferase reporters. These reporters were made using the functional EpRE sequences from GCLC (Fig. 3A) and GCLM (Fig. 3B) and expressed in HEK293A

cells. Overexpression of either p65 Nrf1, p120 Nrf1 or Nrf2 resulted in the increased activity of both reporters above the baseline (Fig. 3A and B), confirming their involvement in mediating EpRE-driven gene expression. Co-transfection of Nrf2 with either Nrf1 forms resulted in diminished EpRE reporter activity to the levels achieved by Nrf1 overexpression alone for GCLC reporter (Fig. 3A) or led to similar result for GCLM reporter,

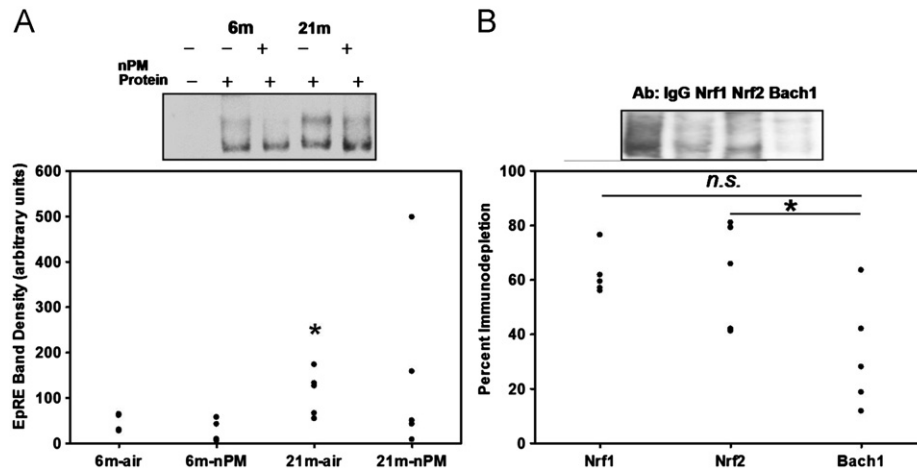


Fig. 4. Age-dependent alteration in protein binding to the GCLM EpRE. Protein extracts from lung samples of young (6-month-old) and middle-aged (21-month-old) mice were analyzed for their ability to bind to a biotinylated EpRE from human *GCLM* promoter with EMSA (gel-shift) assay as described in **Materials and methods**. (A) Age-increased EpRE binding. Representative blot and band densitometry results ($N=5$); *, p -value < 0.05, (Rank sum test). (B) Immunodepletion experiments to determine relative contribution of Nrf1, Nrf2, and Bach1 to the probe. Percentage of immunodepletion (compared to IgG) is shown ($N=5$); *, p -value=0.056, n.s., not significantly different (Rank sum test).

failing to further increase the reporter activity (Fig. 3B). These results suggest dominant role of Nrf1 over Nrf2 and that the former may act as competitive inhibitor of Nrf2/EpRE activity.

Protein binding to the EpRE increases with age

We previously reported that the basal expression level of phase II detoxifying genes was increased in lungs of middle-aged adult (21-month-old) compared to young mice (6-month-old) [14]. To gain insight into the potential role of Nrf1 in this change of EpRE activity with aging, we measured the binding of Nrf1 to GCLM EpRE using gel-shift (EMSA) assays. As shown in Fig. 4A, protein binding to EpRE (manifested as increased band density) was more prominent in lungs of 21-month-old compared to that of 6-month-old mice. There was no obvious effect of nPM exposure notable, except that in the lungs of aged mice no increased EpRE binding was seen. The two age-inducible bands represent the EpRE-protein bound complexes as determined in a separate experiment using non-labeled (“cold”) EpRE probe (data not shown) and as confirmed by immunodepletion experiments discussed below.

Since increased protein binding on EMSA could be due to increased abundance and/or activity of transcriptional activators (e.g., Nrf2) or repressors (e.g., Nrf1 and Bach1), immunodepletion experiments were carried out. All antibodies efficiently depleted the age-inducible EpRE-protein band, but anti-Nrf2 did so with much lower efficiency compared to anti-Bach1 (Fig. 4B), suggesting greater prevalence of Bach1 at the EpRE complex compared to Nrf2.

Nrf1 protein expression is unchanged in aged and nPM-treated animals

Finally, we investigated the effect of age and nPM treatment on Nrf1 protein levels. We estimated the relative migration of full-length and short forms of Nrf1 protein using proteasome inhibitor MG-132, that stabilizes full-length Nrf1 [7,9] and ectopically-expressed Nrf1 p65 as references (Fig. 5A). Mouse short form Nrf1 migrated with an apparent molecular weight of about 7 kDa lower (67 kDa) than human Nrf1 p65 in HEK293A. Such a difference is consistent with previous reports of mouse short form Nrf1 migrating about 10 kDa lower than human short form of Nrf1, as if it were a 55-kDa polypeptide [24] while human

short form Nrf1 resolves at 65 kDa on SDS-PAGE gel [10]. There was no difference in the expression of Nrf1 forms between young and older mice or between unexposed and nPM-exposed mice (Fig. 5B and C).

Discussion

Many studies point to a puzzling observation that the progression of oxidative stress-related, non-malignant human diseases is associated with paradoxical decline in the function of Nrf2 as reviewed elsewhere [25]. It appears that Nrf2 fails to fulfill its cytoprotective function during aging and conditions of elevated oxidative stress when its activity is most needed [25]. Several mechanisms exist to precisely regulate Nrf2 activity as reviewed in [26]. Amongst them is an emerging concept of Nrf1 acting as an inhibitor of Nrf2-mediated transcription [11,12], presumably to limit malignant transformations of healthy cells, associated with Nrf2 overactivation [11,25]. In this study, we tested the hypothesis that Nrf1 may contribute towards previously described impairment of phase II enzyme induction in response to a relevant environmental stressor, nPM [14].

In support of our hypothesis, we found that Nrf1 does indeed appear to act as an inhibitor of Nrf2 in cultured human cells as judged by the results of RNAi experiments (Fig. 2) and luciferase reporter assays (Fig. 3). We note that while the latter provided a conceptually similar conclusion of Nrf2 inhibition by Nrf1, co-transfection of Nrf2 with either Nrf1 forms resulted in diminished EpRE reporter activity (Fig. 3A) or to the lack of further increase (additivity) in the reporter activity (Fig. 3B). Thus, it appears that both Nrf1 and Nrf2 act as activators of the EpRE, but in the presence of both Nrf1 and Nrf2, it is Nrf1 that appears to ultimately determine the magnitude of the EpRE-controlled reporter activity. This is in accordance to the notion [12] that, like Nrf2, Nrf1 regulates EpRE-driven gene expression by association with co-activators other than those of Nrf2 (Fig. 1).

Unlike other EpRE-binding transcription factors Nrf2 and Bach1 [14], we also found that the abundance of Nrf1 fragments remained unchanged in nPM-treated and aged animals (Figs. 4 and 5). This suggests the involvement of other inhibitors (e.g., Bach1 whose protein expression was increased in aged, and even more so, in aged and nPM-treated mice about 2.5- and 3.5-fold, respectively, compared to young, untreated mice) in the

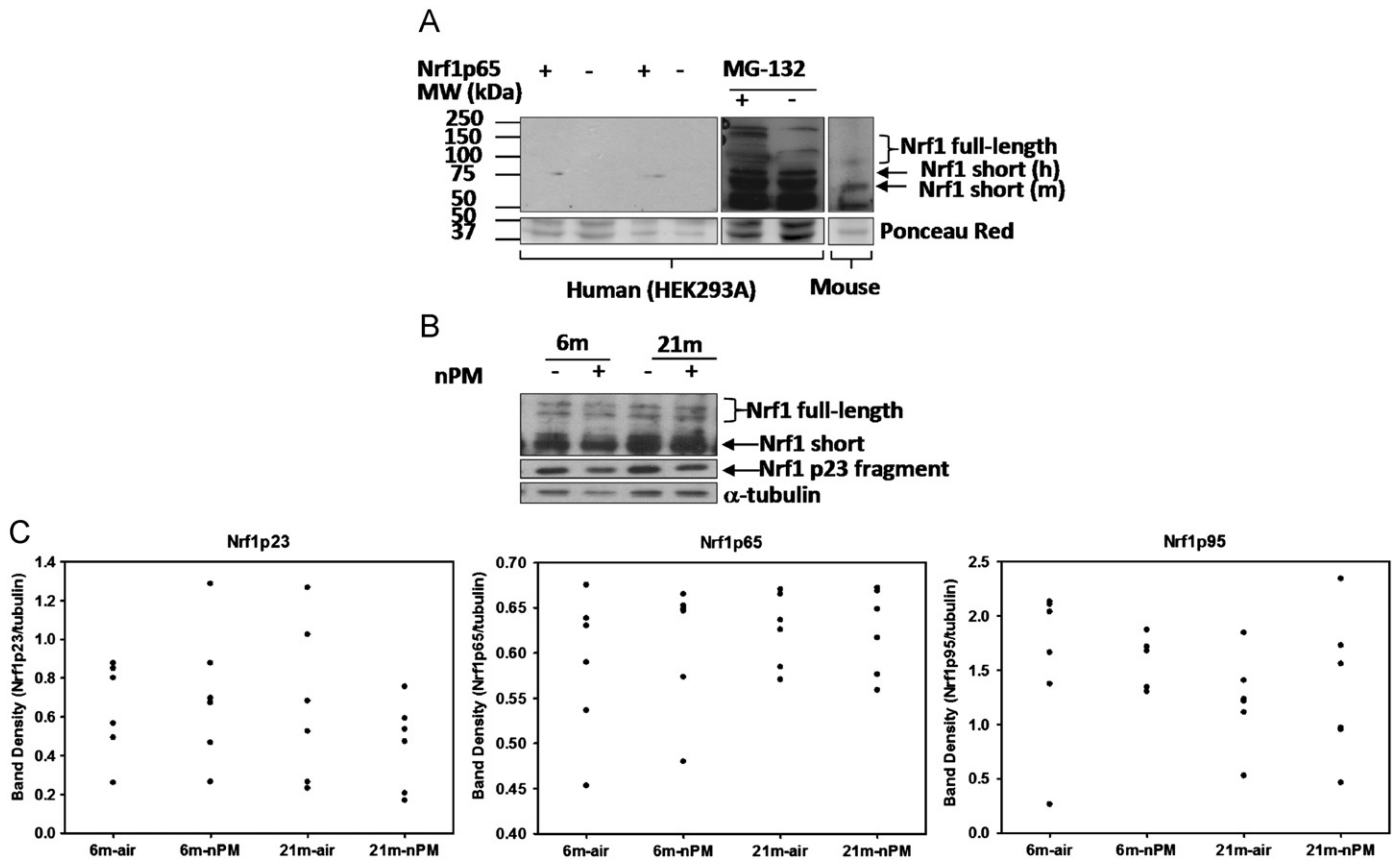


Fig. 5. Nrf1 protein levels remain stable in aged or nPM-treated in mouse lung. Protein extracts from mice, subjected to the ambient air (100% air) or nPM exposure, were immunoblotted as described in Materials and Methods. (A) HEK293 lysates from cells with overexpressed short (Nrf1 p65) form of Nrf1 or MG-132-treated (to stabilize full-length Nrf1) cells were immunoblotted to determine the position of Nrf1 forms in mouse samples. (B) Immunoblotting of mice samples. A representative outcome of six independent experiments is shown. (C) Band densitometry results for Nrf1 immunoblotting ($N=6$). No statistically-significant difference was found between all four conditions for all Nrf1 forms (Rank sum test).

age-dependent impairment of the inducibility of phase II enzymes that we reported recently [14].

Along the same lines, our EMSA results suggest that protein binding to GCLM EpRE increases with age and the EpRE is primarily occupied by EpRE inhibitors Bach1 and Nrf1 rather than activator Nrf2 under basal conditions. This along with increased abundance of Bach1 in aged animals [14] suggest that increased binding to the EpRE in aged animals is mainly due to EpRE inhibitors, which could explain age-dependent loss of inducibility of phase II genes by nPM, reported by our group earlier [14]. This scenario is reminiscent of the displacement of Bach1, bound to the heme oxygenase (HO-1) EpRE and thus inhibiting *HO-1* transcription, by heme; this allows Nrf2 to bind to the EpRE and activate *HO-1* transcription to control heme levels [4].

Conclusions

Taken together, there is accumulating evidence that Nrf1 and especially its short form p65 act as inhibitors of the Nrf2/EpRE pathway. However, direct contribution of Nrf1 to a physiologically-relevant modulation of the Nrf2/EpRE pathway is currently enigmatic. This is the first attempt to address this gap in our knowledge. Our results suggest that Nrf1 does not contribute to age-related decline in the inducibility of phase II enzymes.

Current understanding of the physiological role of Nrf1 in the EpRE-mediated gene expression is greatly impaired by the lack of knowledge on Nrf1 regulation compared to that of Nrf2 (about 50 vs. over 2000 articles up to date, respectively). Potential

limitations of current attempts to characterize Nrf1 function appear to involve differences in competition between Nrf1 and Nrf2 as observed here between cell types and whole lung. These may result from differences in the partner proteins in binding to EpRE. Unlike Nrf2, which robustly responds to various electrophiles in terms of increased protein stability, there has been, to the best of our knowledge, only one report of altered protein expression of Nrf1 in response to a pro-oxidant [27]. In that study, protein expression of Nrf1 in human keratinocytes increased dramatically in response to inorganic arsenite while responding only marginally to known Nrf2 inducers sulforaphane and tert-butylhydroquinone [27]. This suggests that the function of Nrf1 in the EpRE gene expression may be manifested by its altered protein expression only under certain conditions.

It is anticipated that future studies will identify more clearly defined models to study the function of Nrf1 in the EpRE-directed gene expression. In addition, Nrf1 regulation at the protein level needs further clarification. For instance, immunoprecipitation followed by mass spectrometry can aid in the identification of the site(s) of proteolytic cleavage of Nrf1 protein and in documenting the suite of Nrf1- and Nrf2-associated co-activators and inhibitors to explain the competition of Nrf1 with Nrf2 in mediating expression of the EpRE-driven gene expression.

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