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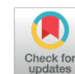
Silencing Bach1 alters aging-related changes in the expression of Nrf2-regulated genes in primary human bronchial epithelial cells



Hongqiao Zhang[a,*], Lulu Zhou[a], Kelvin J.A. Davies[a,b,c], Henry Jay Forman[a]

[a] Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA90089, United States
[b] Division of Molecular & Computational Biology, Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, University of Southern California, United States
[c] Department of Biochemistry & Molecular Medicine, Keck School of Medicine of USC, University of Southern California, United States

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ABSTRACT

Nrf2 is the master transcription factor regulating the basal and inducible expression of antioxidant genes. With aging, the basal Nrf2 activity is increased but oxidant/electrophile-enhanced activation of Nrf2 signaling is diminished, and these changes are accompanied by an increased expression of Bach1, a repressor of Nrf2 signaling. In this limited follow-up study, we explored how Bach1 may be involved in aging-related alteration in Nrf2 signaling in primary human bronchial epithelial (HBE) cells. Silencing Bach1 with siRNA increased the basal mRNA expression of Nrf2 regulated genes including glutamate cysteine ligase catalytic (GCLC) and modifier subunit (GCLM), NAD(P)H oxidoreductase 1(NQO-1) and heme oxygenase 1(HO-1), in HBE cells from both young (aged 21–29 years) and older (aged 61–69 years) donors. On the other hand, Bach1 silencing affected the induction of Nrf2-regulated genes differentially in young and older HBE cells. Bach1 silencing significantly enhanced sulforaphane-induced expression of HO-1 but had no effect on that of GCLC, GCLM, and NQO1 in young HBE cells. In contrast, Bach1 silencing enhanced sulforaphane-induced expression of GCLC, GCLM and HO-1 but had no effect on that of NQO-1 in older HBE cells. In conclusion, these results suggest that increased Bach1 contributes to aging-related loss of electrophile-enhanced Nrf2 signaling.

1. Introduction

Nuclear factor E2-related factor 2 (Nrf2) is the master transcription factor that regulates the expression of a battery of hundreds of antioxidant/detoxifying genes [1]. One of the most important features of the Nrf2-regulated antioxidant system is its inducibility in response to oxidative stress, via the activation of Nrf2. Under basal condition, Nrf2 activity in the nucleus remains low due to constant degradation of Nrf2 by 26S proteasomes in the cytosol facilitated by Keap1, an adaptor of E3-ubiquitin ligase. Under oxidative stress, Keap1 is inactivated due to oxidative modification and thus Nrf2 is dissociated from Keap1 [2,3]. In addition, oxidants induce dissociation of 26S proteasomes into free 20S proteasomes (that can degrade oxidized proteins) and 19S activators, in a process catalyzed by ECm29 and HSP70 [4]. The combination of Keap1 inactivation and 26S proteasome dissociation allows Nrf2 to escape degradation [2–4]. Activated Nrf2 enters the nucleus, binds to the electrophile response element (EpRE, also called the antioxidant response element) in the promoters of target genes, and increases gene transcription [2]. Nrf2 signaling; i.e., Nrf2 activation and induction of the Nrf2-regulated antioxidant/detoxifying genes, boosts antioxidant capacity and enables cells to maintain redox homeostasis and protect against injuries from oxidative insults. Thus, Nrf2 activation is a critical mechanism of antioxidant defense.

With aging, Nrf2 signaling is altered [2]. Reports on the effect of aging on basal Nrf2 activity (marked by nuclear Nrf2 protein, EpRE binding activity and expression of Nrf2-regulated antioxidant genes) in tissues/cells from older individuals were either decreased [5–8], unchanged [9], or increased [10–13], compared to that in young adults. These differential effects of aging on basal Nrf2 activity may be attributable to the variety of ages, tissues, cells, and species used in these studies. On the other hand, mounting evidence suggest that oxidant/electrophile-enhanced Nrf2 activation and induction of Nrf2-regulated antioxidant genes above baseline declines with aging [6,7,11–14]. In previous studies in mice and primary human bronchial epithelial (HBE) cells, we demonstrated that the basal Nrf2 activity was increased but the enhancement of Nrf2 activation by ambient nanoparticles or

* Corresponding author. Leonard Davis School of Gerontology, University of Southern California, 3715 McClintock Avenue, Los Angeles, CA, 90089-0191, United States.
E-mail address: hongqiaz@usc.edu (H. Zhang).

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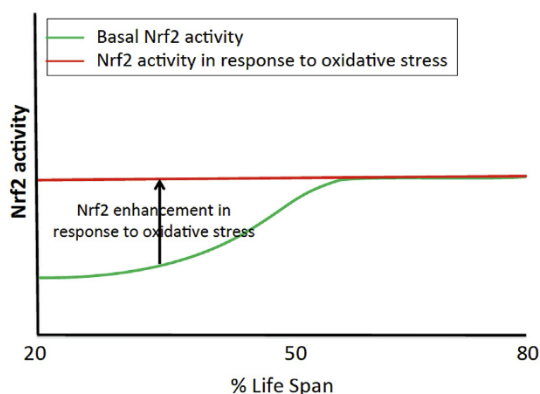


Fig. 1. Hypothetic model: aging-related changes of Nrf2 activation. With aging, the basal Nrf2 signaling is increased but the enhancement of Nrf2 activation by oxidant/electrophile reaches a ceiling and cannot be further increased.

sulforaphane was impaired in older versus younger adults [11–13]. Based on these findings we propose a model of aging effects on Nrf2 signaling (Fig. 1). In this model, the basal Nrf2 signaling is gradually increased with aging due to aging-related increase in oxidant production, until reaching a plateau at which there is no further Nrf2 activation in response to oxidative challenge. The aging-related loss of oxidant/electrophile-enhanced Nrf2 activation impairs the adaptation to and defense against further oxidative stress, thereby making the elderly vulnerable to oxidative stress-associated diseases. However, the underlying mechanism by which aging impairs Nrf2 signaling remains uncertain.

Nrf2 signaling is regulated by many factors, including those involved in Nrf2 protein stability, phosphorylation, nuclear exportation, and formation of Nrf2-EpRE complex [2]. In the nucleus, Nrf2 activity is regulated by several factors including Bach1 [2]. Bach1 belongs to the basic region leucine zipper protein family and is a transcription factor ubiquitously expressed [15–17]. By competing with Nrf2 for forming heterodimers with Maf proteins and then binding EpRE, Bach1 down regulates Nrf2-regulated antioxidant genes [18,19], as demonstrated in studies on Bach1 silencing cells and mice [20,21]. With aging, Bach1 expression is increased [11–13], but how increased Bach1 is involved in aging-related alteration of Nrf2 signaling has, thus far, remained unclear. In this study, we investigated the role of Bach1 in aging-related changes in Nrf2 signaling using primary HBE cells from young and older donors. In the cells from the latter, Bach1 expression was increased and sulforaphane-enhanced Nrf2 activation decreased [13]. Here, our data showed that silencing Bach1 not only increased the basal expression of Nrf2-regulated antioxidant genes, but also partially reversed the aging-related decline in electrophile-enhanced Nrf2 signaling.

2. Materials and methods

2.1. Reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO, USA). Negative control and Bach1 siRNAs were from Santa Cruz (Santa Cruz, CA, USA). RNA extraction kit was from Qiagen (Qiagen Inc, CA, USA). TRIzol reagent, Reverse Transcription kit, SYBR Green PCR master mixture, Lipofectamine 2000, and DNA-free reagent were from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL, USA).

2.2. Cell culture and treatment

Primary HBE cells were obtained from Lonza (Lonza, MD, USA). Donor information were as described before [22]. Cells were cultured on collagen coated flasks in BEGM basal medium (Lonza, MD, USA) supplemented with cell growth factors at 37 °C in 5% CO_2 incubator. All the experiments were conducted using cells with less than 8 passages. HBE cells were seeded in 6 well plates. At 70% confluence, cells were transfected with 50 nM of Bach1 siRNA with lipofectamine 2000 for 24 h. Cells were recovered by replacing with fresh medium for 7 h, and then treated with sulforaphane at 5 μM for 18 h.

2.3. Quantitative PCR assay

RNA was extracted with RNeasy mini kit or TRIzol reagent following the protocol provided by the manufacturer. Total RNA was treated with DNA-free reagent to remove genomic DNA contamination. After reserve transcription, mRNA was determined by quantitative PCR. Sequences of primers targeting β-actin, Bach1, GCLC, GCLM, HO-1 and NQO1 were as reported before [13,23].

2.4. Statistical analysis

A comparative ΔΔCT method was used for the relative mRNA quantitation. Data were expressed as mean ± standard error. Prism 7 was used for statistical analysis, and statistical significance was accepted at $p < 0.05$. Non-parametric ANOVA equivalent (Kruskall-Wallis) was used for analysis of significant effect of treatment on mRNA levels.

3. Results

3.1. Silencing Bach1 increased the basal expression of Nrf2-regulated antioxidant genes in both young and older HBE cells

To study how Bach1 is involved in aging-related alteration of Nrf2 signaling, we first examined the effect of Bach1 silencing on the expression of Nrf2-regulated gene expression, including glutamate cysteine ligase catalytic and modifier subunits, (GCLC and GCLM, respectively), NAD(P)H oxidoreductase 1(NQO-1), and heme oxygenase 1 (HO-1). Bach1 expression was significantly decreased by 50–60% after Bach1 siRNA transfection in both young and older primary HBE cells (Fig. 2). Bach1 silencing increased the expression of the four representative Nrf2-regulated antioxidant genes (GCLC, GCLM, NQO-1 and HO-1) in HBE cells from both young (aged 21–29 years) and older (aged 61–69 years) donors (Fig. 3). The Bach1 silencing-caused increase in the expression of Nrf2-regulated genes was less in the HBE cells from

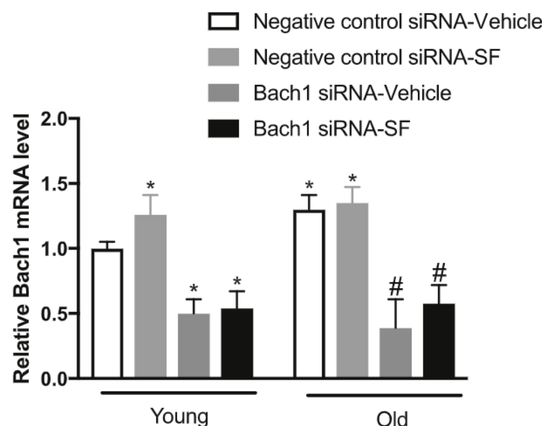
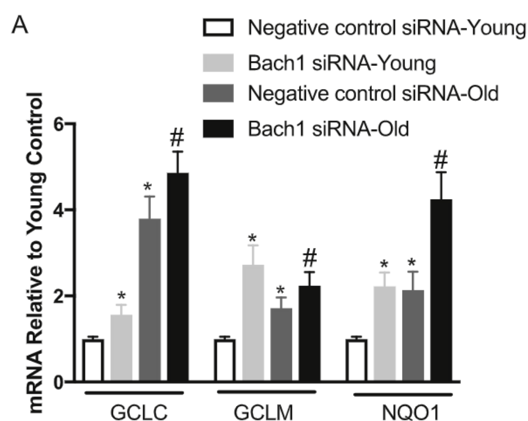


Fig. 2. Silencing Bach1 with siRNA. HBE cells from young and older donors were transfected with negative control or Bach1 siRNA (50 nM) for 24 h and then recovered in fresh medium for 7 h before being treated with/without sulforaphane (5 μM) for 18 h Bach1 mRNA was determined with quantitative PCR. *, $P < 0.05$ compared with negative control siRNA-vehicle of cells from the young donors; #, $P < 0.05$ compared with negative control siRNA-vehicle of cells from older donors, N = 3.



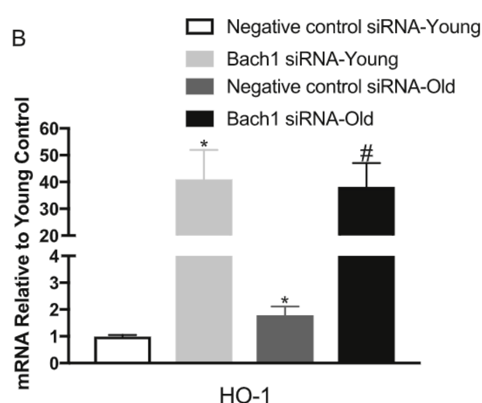


Fig. 3. Silencing Bach1 increased mRNA expression of Nrf2-regulated genes in HBE cells from both young and old donors. Cells were transfected with negative control or Bach1 siRNA (50 nM) for 24 h and then recovered in fresh medium for 25 h. The mRNA was determined with quantitative PCR. (A) mRNA of GCLC, GCLM, and NQO-1. (B) HO-1 mRNA. *, $P < 0.05$ compared with negative control siRNA-vehicle of cells from the young donors; #, $P < 0.05$ compared with negative control siRNA-vehicle of cells from older donors, N = 3.

older versus young donors. In addition, as previously reported [13], the basal mRNA expression of Nrf2-regulated genes was significantly higher in the HBE cells from older versus young donors. Compared to other genes, HO-1 expression was the most greatly influenced by Bach1 silencing, and it was increased by about 40-fold with Bach1 silencing, compared to a maximum of less than 4-fold of other three genes (GCLC, GCLM and NQO1). These data suggest that Bach1 functions as Nrf2 signaling suppressor even under basal condition and that inhibition of Bach1 could up-regulate basal Nrf2 signaling.

3.2. Silencing Bach1 restored aging-related loss of sulforaphane-enhanced Nrf2 activation

To examine how Bach1 was involved in aging-related decline in oxidant/electrophile-enhanced Nrf2 activation, we determined the induction of Nrf2-regulated antioxidant genes in HBE cells from and older donors with/without Bach1 silencing by sulforaphane, a potent activator of Nrf2 signaling [13]. Treatment of HBE cells with sulforaphane for 18 h increased the mRNA expression of the four representative Nrf2-regulated genes (Fig. 4). Similar to previous report [13], the induction of Nrf2-regulated genes in the HBE cells from older individuals was significantly lower than that seen in cells from younger donors. Bach1 silencing with siRNA, which increased the basal expression of Nrf2-regulated genes (Fig. 3), did not significantly change the mRNA induction of Nrf2-regulated genes by sulforaphane in the young HBE cells, except HO-1, which induction was significantly enhanced with Bach1 silencing in cells from young donors. In contrast, in HBE cells from older donors, silencing Bach1 increased the induction of GCLC, GCLM and HO-1 significantly compared to cells transfected with negative control siRNA (Fig. 4). However, Bach1 silencing did not affect the induction of NQO-1 in cells from older individuals. These data suggest that silencing Bach1 restored aging-related decline in electrophile-enhanced Nrf2 signaling in a gene-dependent manner.

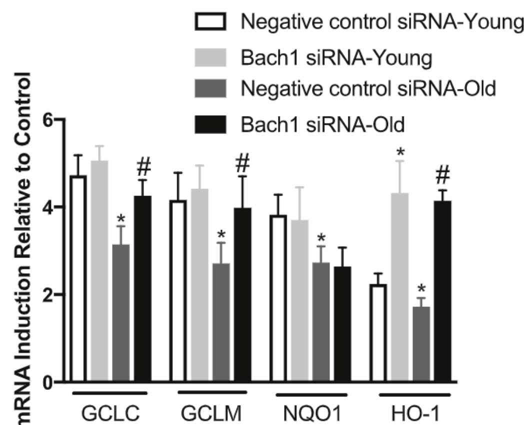


Fig. 4. Silencing Bach1 restored mRNA induction of Nrf2-regulated genes by sulforaphane in HBE cells from older donors. HBE cells from young and older donors were transfected with negative control or Bach1 siRNA (50 nM) for 24 h and then recovered in fresh medium for 7 h before being treated with/without sulforaphane (5 μM) for 18 h. The mRNA was determined with quantitative PCR. *, $P < 0.05$ compared with vehicle control; #, $P < 0.05$ compared with negative control siRNA-vehicle control of cells from older donors, N = 3.

4. Discussion

Accumulating evidence suggest that oxidant/electrophile-enhanced Nrf2 activation declines with aging. The results here partially clarify the underlying mechanism. Using primary HBE cells from young and older donors, the current study showed that silencing Bach1 enhanced sulforaphane-induced expression of Nrf2-regulated genes in HBE cells from older donors, consistent with the hypothesis that an increase in Bach1 with aging [11–13] underlies the aging-related decline in oxidant/electrophile-enhanced Nrf2 activation.

Several studies have demonstrated that Bach1 suppresses Nrf2 signaling by competing with Nrf2 for dimerization partners and for the binding of EpRE sites in the promoter of target genes [17–20]. Inhibition of Bach1, either through gene knockout [21,24], or through pharmacological inhibitors [18,20,25–29], enhanced the expression of Nrf2-regulated genes particularly HO-1. We observed a consistent and significant increase in the basal expression of the four representative Nrf2-regulated genes after silencing Bach1 in HBE cells from both young and older donors (Fig. 3). These data indicate that under basal condition, Bach1 functions to suppress Nrf2 signaling in cells from both young and older individuals. More importantly, the basal expression of Nrf2-regulated genes, which was increased in the elderly and could not be efficiently induced by Nrf2 activator [11–13], was up regulated via Bach1 silencing, suggesting that Bach1 acts as a brake to blunt Nrf2 signaling in older individuals, especially considering that nuclear Bach1 protein is increased in the elderly [11–13]. It also suggests that the ceiling effect of aging on Nrf2 signaling (Fig. 1) can be breached. It remains to be determined whether the increased basal expression of these Nrf2-regulated genes is due to less Bach1 binding, or also requiring a subsequent increase in Nrf2 binding to the same EpRE of target genes.

As previously reported [13], the induction of the four representative Nrf2-regulated genes by sulforaphane was significantly reduced in HBE cells from older versus younger individuals (Fig. 4). Silencing Bach1 enhanced the induction of three of the four genes, i.e., GCLC, GCLM and HO-1, in HBE cells from older donors (Fig. 4), suggesting that Bach1 is responsible for aging-related decline of oxidant/electrophile-enhanced induction of these genes. In contrast, Bach1 silencing in cells from older donors did not further enhance NQO-1 induction by sulforaphane. While in HBE cells from younger donors, silencing Bach1 enhanced the induction of only HO-1 but not other three genes by sulforaphane. This evidence suggests that the regulatory effects of Bach1 on Nrf2 signaling are gene dependent. Others have also observed this gene-dependent effect of Bach1. For example, Reichard et al. found that Bach1 inactivation was necessary for HO-1 induction, but not for the induction of thioredoxin reductase 1, another Nrf2-regulated gene, in HaCat human keratinocytes [20]. This gene specific regulation can be partially explained by EpRE sequence dependent binding of different transcription factors. EpRE can be bound by various heterodimeric basic leucine zipper (bZip) factors including Nrf1, Nrf2, Nrf3, Bach-1, Maf and AP-1 families [17,30–36], and the composition of the heterodimers determines they are transcription activators or suppressors. In addition, the binding of these heterodimers to EpREs of target genes is sequence dependent [37]. Although EpRE core sequences (TGAnnnnGC) is same for most Nrf2-regulated genes, the internal and flanking nucleotides of the EpREs vary with genes and this can lead to the binding of different heterodimers. For example, C3 in the 5′-end of the flanking region of EpRE consequence was required for EpRE activation of NQO-1 [38], but not for EpRE4 activation of GCLC [37], and human HO-1 is regulated by multiple EpREs in its promoter [1,39–41]. The above evidence may help to explain the different effects of silencing Bach1 on the induction of HO-1 and NQO-1 in HBE cells from young and older individuals respectively. Nonetheless, the key finding here is that Bach1 is involved in aging-related decline in Nrf2 activation.

Although Nrf2 signaling is negatively regulated by Bach1, the increase in the expression of Nrf2 target genes after Bach1 silencing is not a linear function of Bach1 reduction (Fig. 2). As discussed above, the regulation of Nrf2-target genes is complicated and other factors than Nrf2/EpRE signaling may also be involved, including 1) multiple EpREs in one promoter, not all of which may bind Bach1 2) the AP-1 site embedded in the EpRE may be functional, 3) other cis-elements contribute to transcriptional regulation of genes that are regulated through EpREs, and 4) variation in Nrf2 partners can up or downregulate transcription mediated through EpREs. These factors lead to the variation in transcription among Nrf2 regulated genes, and different transcription machinery of the same gene after Bach1 silencing. The possibility also exists that proteins other than Bach1 interfere with Nrf2 function in the nucleus. Therefore, although Bach 1 expression was decreased by 50–60% in both young and older HBE cells, one would actually expect a non-linear increase in Nrf2 target gene expression as a function of Bach 1 reduction, as was observed (Fig. 2). Determination or which of these or other factors that account for the non-linearity is well beyond the scope of the current study.

In summary, we found that silencing Bach1 increased the basal expression of Nrf2-regulated genes and restored aging-related decline in the induction of these genes by Nrf2 activator in primary HBE cells. The results indicate that Bach1 contributes to aging-related decline in Nrf2 signaling and suggest the potential use of Bach1 as an intervention target to modulate Nrf2 signaling in cells/tissues that express an increased nuclear Bach1 and the accompanying age-related loss of Nrf2 activation in older individuals. The restoring effect of Bach1 inhibition on the induction of Nrf2-regulated genes in HBE cells from older donors was gene-dependent, suggesting that besides Bach1, other factors are also involved in the aging-related decline in Nrf2 activation. Further studies will now be required to expand our understanding of the effects of Bach1 inhibition on the induction of other Nrf2-regulated genes, and on the composition of transcription factors binding to different EpRE sequences in different genes. Future studies will also examine the functional significance of age-related change in Nrf2 signaling (the increase in basal activity and the decline in Nrf2 activation), and determine whether Bach1 silencing/inhibition can increase the protection against oxidative stress in the older individuals as expected.

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