Heme Oxygenase-1 Protects Human Melanocytes from H_2O_2-Induced Oxidative Stress via the Nrf2-ARE Pathway

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Oxidative stress caused by hydrogen peroxide (H_2O_2) leads to cell death and has been implicated in the pathogenesis of vitiligo. The nuclear factor E2-related factor 2 (Nrf2)-antioxidant response element (ARE), a major antioxidant pathway, regulates oxidative stress-related cytoprotective genes. We hypothesized that the Nrf2-ARE pathway protects human melanocytes from H_2O_2-induced oxidative damage through the induction of downstream antioxidative genes. Thus, we used Nrf2 short interfering RNA (siRNA) and pCMV6-XL5-Nrf2 to downregulate or upregulate Nrf2 expression in immortalized human melanocyte cell line PIG1. The melanocytes were then analyzed under different oxidative stress conditions for cell viability and apoptosis. Our study demonstrated that *heme oxygenase-1* (*HO-1*) was the most induced antioxidant gene in PIG1 cells after treatment with H_2O_2. Knockdown of Nrf2 or zinc protoporphyrin IX (ZnPP) treatment increased cell death caused by H_2O_2 in melanocytes, but upregulation of Nrf2 or hemin treatment reduced cell death caused by H_2O_2 in melanocytes. In addition, the H_2O_2-induced Nrf2-ARE/HO-1 pathway was confirmed in primary cultured human melanocytes by examining the expression and translocation of Nrf2 and HO-1. These data suggested that regulation of the Nrf2/HO-1 pathway can reduce H_2O_2-induced oxidative damage in human melanocytes. Our data demonstrate that HO-1 protects human melanocytes from oxidative damage via the Nrf2-ARE pathway.

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INTRODUCTION

Human skin is a major target of oxidative stress because of multiple endogenous or exogenous insults by an array of chemical and physical agents. These environmental toxicants or their metabolites can directly and indirectly drive the production of reactive oxygen species (ROS) (Trouba *et al.*, 2002; Bickers and Athar, 2006). ROS may be involved in the pathogenesis of numerous skin disorder diseases, including the acquired skin depigmentation disorder, vitiligo (Bickers and Athar, 2006). Among a great variety of ROS, hydrogen peroxide (H_2O_2) has a pivotal role because it is generated from nearly all sources of oxygen radicals (Lee and Shacter, 2000; Barbouti *et al.*, 2002). Although the pathogenesis of vitiligo is still unclear, increasing evidence shows that oxidative stress caused by H_2O_2 is a key factor in the onset and progression of this disease (Schallreuter *et al.*, 2007).

Fortunately, the skin has its own antioxidant system. Nuclear factor E2-related factor 2 (Nrf2) is a critical transcription factor in protecting cells from oxidative damage (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004). Recently, the functions of Nrf2 and its downstream genes have been shown to be important for protection against oxidative stress and chemical-induced cellular damage in many organs and diseases (Chan *et al.*, 2001; Enomoto *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Braun *et al.*, 2002; Fahey *et al.*, 2002), but only a few studies have focused on vitiligo or melanocytes. Moreover, *Nrf2* gene polymorphisms in the A^{-650} allele may be a risk factor associated with the development of vitiligo, which implies that the Nrf2-associated antioxidant process has an active role in the pathogenesis of vitiligo (Guan *et al.*, 2008). Moreover, Nrf2 is involved in the human skin cell pathways and ensures protection of skin against photo-oxidative stress (Marrot *et al.*, 2008).

The Nrf2-antioxidant response element (ARE) may be the most important pathway in protecting cells from oxidative stress (Kaspar *et al.*, 2009), and it is also involved in the pathways of human skin adaptation to environmental stress (Marrot *et al.*, 2008). To our knowledge, only a limited

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Abbreviations: ARE, antioxidant response element; GCLC, γ-glutamyl cystine ligase catalytic subunit; GCLM, γ-glutamyl cystine ligase modulatory subunit; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; NQO-1, NAD(P)H: quinone reductase; Nrf2, nuclear factor E2-related factor 2; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, real-time PCR; siRNA, short interfering RNA; ZnPP, zinc protoporphyrin IX

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number of studies have focused on human melanocytes to determine the antioxidative role of the Nrf2-ARE pathway and to investigate the expression of phase II genes. Therefore, we hypothesized that the Nrf2-ARE pathway protects human melanocytes from apoptotic and necrotic death caused by H_2O_2 and that the change of heme oxygenase-1 (HO-1) activity may serve as a key mechanism of cytoprotection during H_2O_2-mediated oxidative stress.

To test our hypothesis, we downregulated and upregulated Nrf2 to elucidate the antioxidative effects of the Nrf2-ARE pathway, and we also investigated the role of HO-1 in cell viability of human melanocytes using zinc protoporphyrin IX (ZnPP), a specific inhibitor of HO-1, and hemin, an activator of HO-1. In this study, we demonstrated that Nrf2-driven transcriptional activation of ARE increases the generation of HO-1, which has a crucial role in protecting human melanocytes from H_2O_2-induced oxidative damage.

RESULTS

Downregulation of Nrf2 increases sensitivity to oxidative stress, but upregulation of Nrf2 protects melanocytes from H_2O_2 toxicity

To investigate the role of Nrf2 in the susceptibility of melanocytes to H_2O_2-mediated oxidative stress, we used Nrf2 short interfering RNA (siRNA) or pCMV6-XL5-Nrf2 to downregulate or upregulate, respectively, the expression of Nrf2. Figure 1a shows the real-time PCR (RT-PCR) results, which indicate that the level of Nrf2 mRNA in PIG1 cells was reduced to <70% of the control at 48 hours after Nrf2 siRNA treatment. Decreased Nrf2 protein levels were observed 72 hours after transfection (Figure 1b). Cells treated with either mock or scrambled siRNA samples had no change in Nrf2 mRNA and protein expression levels. In contrast, significantly increased mRNA and protein levels of Nrf2 were observed in PIG1 cells transfected with pCMV6-XL5-Nrf2 for 48 or 72 hours, and these changes were not observed in mock-treated cells (pCMV6-XL5; Figure 1c and d). In subsequent experiments, PIG1 cells were pretreated with different agents (pCMV6-XL5, pCMV6-XL5-Nrf2, scrambled siRNA, or Nrf2 siRNA3) for 48 hours. An additional 24 hours after exposure to 1.0 mM H_2O_2, an optimal concentration that would lead to a consistent and high degree of cytotoxicity as concluded in our preliminary investigation (data not shown), PIG1 cell viability was examined by MTS assay. As shown in Figure 1e, suppressed Nrf2 expression led to significant cell death (>75%) under oxidative stress, and the percentage of dead cells was low in cells with upregulated Nrf2 expression (11.8%).

To further understand if the reduction in cell viability was because of apoptosis, we next performed flow cytometry with annexin V and propidium iodide staining to assess the apoptosis rates in melanocytes transfected with Nrf2 siRNA or pCMV6-XL5-Nrf2 before the cells were exposed to 1.0 mM H_2O_2. Compared with the control group, the proportion of apoptotic and necrotic cells increased after Nrf2 was downregulated by Nrf2 siRNA, but there was a statistically significant induction in apoptosis 48 hours after transfection

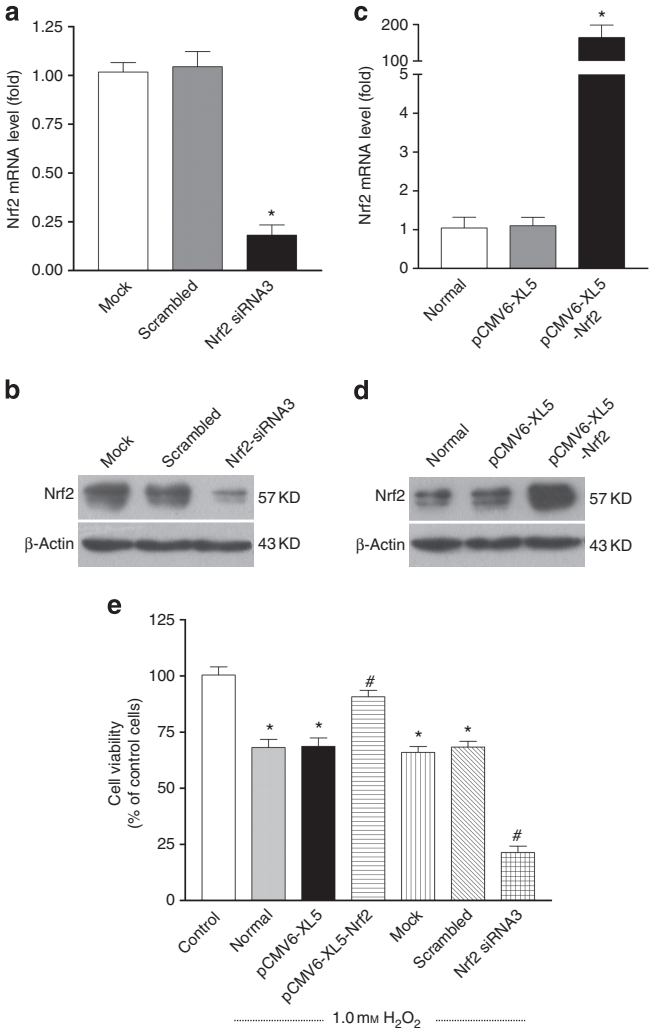

Figure 1. Analysis of nuclear factor E2-related factor 2 (Nrf2) levels and cell viability in PIG1 cells after transfection with Nrf2 siRNA3 or pCMV6-XL5-Nrf2. Nrf2 (**a**) mRNA and (**b**) protein levels were measured by real-time PCR (RT-PCR) and western blots after transfection with Nrf2 siRNA3. *P<0.01 compared with mock transfection. Nrf2 (**c**) mRNA and (**d**) protein levels were measured by RT-PCR and western blots after transfection with pCMV6-XL5-Nrf2. *P<0.01 compared with normal. β-Actin was used as an internal control. (**e**) Cells were pretreated with pCMV6-XL5-Nrf2 or Nrf2 siRNA3. Cell viability was determined with MTS assay 24 hours after exposure to 1.0 mM hydrogen peroxide (H_2O_2). #P<0.01 compared with normal (mock transfection group treated with H_2O_2). *P<0.01 compared with control (untreated).

with pCMV6-XL5-Nrf2 (38.7 vs. 4.7%; 3.5% vs. 0.7%; and 1.1 vs. 6.1%, respectively; Figure 2).

HO-1 is the main target gene of the Nrf2-ARE pathway in protecting human melanocytes against oxidative stress

To determine which downstream gene of the Nrf2-ARE pathway was the most important antioxidative gene, we examined the mRNA levels of four main phase II detoxifying enzymes including *NAD(P)H: quinone reductase* (*NQO-1*), *γ-glutamyl cystine ligase catalytic subunit* (*GCLC*), *γ-glutamyl cystine ligase modulatory subunit* (*GCLM*), and *HO-1*. Figure 3a shows the expression of the four main phase II

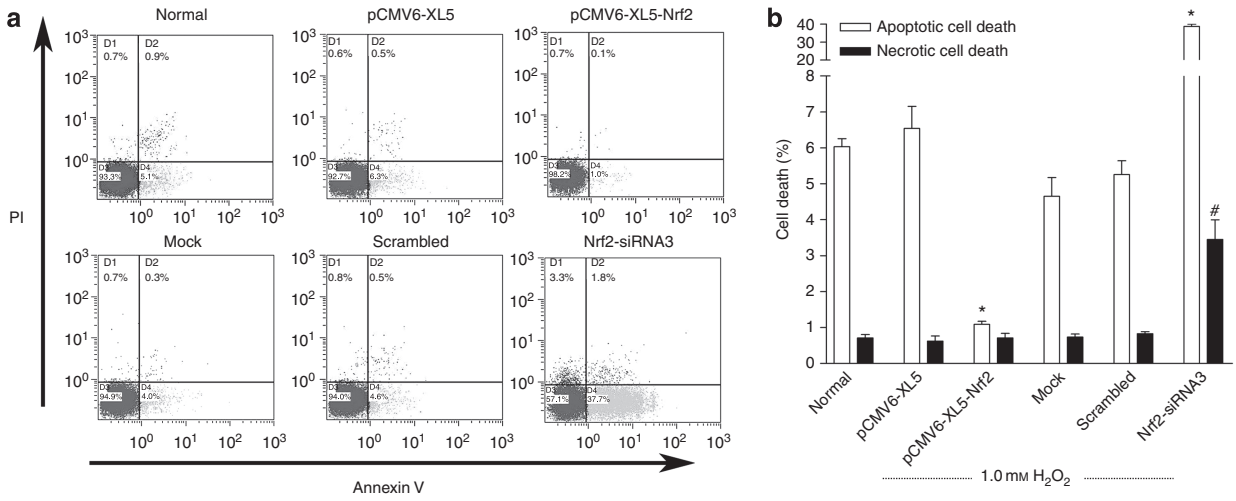

Figure 2. Determination of apoptosis for cells pretreated with pCMV6-XL5-Nrf2 or nuclear factor E2-related factor 2 (Nrf2) short interfering RNA3 (siRNA3) at 24 hours after exposure to 1.0 mM hydrogen peroxide (H_2O_2). H_2O_2 led to both apoptosis and necrosis in melanocytes. Knockdown of Nrf2 caused more H_2O_2-induced apoptotic and necrotic cell death when compared with normal cells (untransfected). Upregulation of Nrf2 caused less H_2O_2-induced apoptotic cell death when compared with normal cells. (**a**) Cells were stained with annexin V and propidium iodide (PI) for 15 minutes and analyzed by flow cytometry (FCM). Representative FCM graphs are shown. (**b**) Bar graphs represent the mean values of FCM data ($n=3$). $^{\#}P<0.05$, $^{*}P<0.01$ when compared with normal cells.

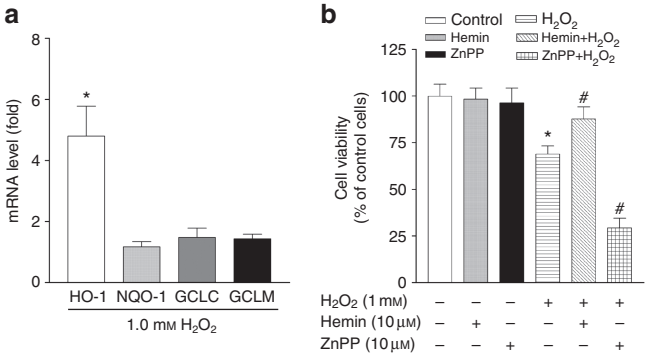

Figure 3. Investigation of phase II gene modulation and the protective effect of heme oxygenase-1 (HO-1). (**a**) Modulation of *HO-1*, *NQO-1*, *GCLC*, and *GCLM* gene expression levels was detected by real-time PCR (RT-RCR) in PIG1 melanocytes after a 24-hour hydrogen peroxide (H_2O_2) treatment. Data are shown as ratios of gene expression levels in treated cells to that in untreated controls after normalization based on the expression of the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene. *GCLC, γ-glutamyl cystine ligase catalytic subunit; GCLM, γ-glutamyl cystine ligase modulatory subunit; NQO-1, NAD(P)H: quinone reductase.* (**b**) Viability of melanocytes pretreated with hemin or zinc protoporphyrin IX (ZnPP; 10 μM) was determined with MTS assay 24 hours after exposure to 1.0 mM H_2O_2 ($n=5$). $^{*}P<0.01$ compared with the control cells. $^{\#}P<0.01$ compared with the H_2O_2 treatment group.

detoxifying genes in melanocytes after a 24-hour treatment with 1.0 mM H_2O_2. The RT-PCR results are shown in Figure 3a and demonstrate that the expression levels of all four genes were upregulated. The most significant change was found in the *HO-1* gene with ~5-fold increase when compared with the control.

To determine whether the increased expression of HO-1 was responsible for the cytoprotective effects against H_2O_2-derived oxidative cell death, the HO-1 specific inhibitor, ZnPP, and activator, hemin, were utilized in this study. Treatment with ZnPP or hemin alone at concentrations of 10 μM for 24 hours did not affect cell viability (96.3 ± 8.7 and 98.3 ± 5.9% of the control value, respectively; $n=5$). Interestingly, ZnPP exacerbated the damage (29.2 ± 5.2% of the control value) induced by H_2O_2 in melanocytes, and hemin enhanced cells viability to 87.7 ± 6.5% compared with the control (Figure 3b).

The expression of HO-1 follows the Nrf2-dependent manner in human melanocytes

To determine the role of Nrf2 in H_2O_2-induced HO-1 expression, we treated melanocytes with Nrf2 siRNA or pCMV6-XL5-Nrf2. As mentioned above, significant changes occurred in Nrf2 mRNA and protein expression levels at 48 and 72 hours after transfection of these constructs (Figure 1). The reduction of HO-1 mRNA expression was associated with Nrf2 knockdown when compared with the cells treated with mock and scrambled siRNA (Figure 4a). Similarly, Nrf2 knockdown decreased HO-1 protein expression levels (Figure 4b). The increase in both mRNA and protein levels of HO-1 coincided with increased Nrf2 levels, but no changes in HO-1 expression levels were found in untreated cells or in pCMV6-XL5-treated cells (Figure 4c and d).

H_2O_2 induces the Nrf2-ARE/HO-1 pathway in primary cultured human melanocytes

To determine whether the Nrf2-ARE/HO-1 pathway is induced by H_2O_2-mediated damage in normal primary human melanocytes, RT-PCR analysis and immunocytochemistry were carried out to examine the expression of Nrf2 and HO-1 in the normal primary human melanocytes after the treatment with H_2O_2. Figure 5a shows the expression of Nrf2 and HO-1 mRNA levels in primary normal human melanocytes after a 24-hour treatment with 1.0 mM H_2O_2. The mRNA levels of Nrf2 and HO-1 were significantly higher in H_2O_2-treated groups when compared with untreated groups. Immunocytochemistry analysis

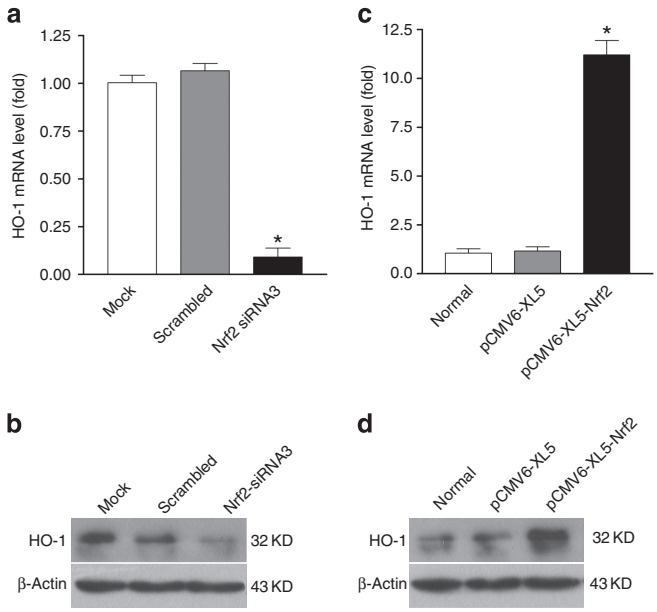

Figure 4. Analysis of heme oxygenase-1 (HO-1) mRNA and protein levels after transfection with nuclear factor E2-related factor 2 (Nrf2) siRNA3 or pCMV6-XL5-Nrf2 in PIG1 cells. (**a**) Real-time PCR (RT-PCR) results demonstrated decreased HO-1 mRNA levels in the Nrf2 siRNA3 sample when compared with the mock sample 48 hours after transfection. *$P<0.01$ compared with the mock transfection by Student's *t*-test. (**b**) Western blot analysis demonstrated lower HO-1 protein expression 72 hours post-transfection when compared with the mock sample. (**c**) RT-PCR results demonstrated increased HO-1 mRNA levels in the pCMV6-XL5-Nrf2 sample when compared with the control sample 48 hours after transfection. *$P<0.01$ compared with normal cells. (**d**) Western blot analysis demonstrated higher HO-1 protein expression 72 hours post-transfection when compared with control cells. β-Actin was used as an internal control.

showed that the fluorescent intensities of Nrf2 and HO-1 in the H_2O_2-treated groups were greater than that in untreated groups (Figure 5b and c). Furthermore, a large proportion of Nrf2 and a small proportion of HO-1 proteins moved into the nucleus after treatment with 1.0 mM H_2O_2 (Figure 5b and c). These results indicate that H_2O_2 treatment increased the expression and nuclear translocation of Nrf2 and induced HO-1 expression in normal primary human melanocytes.

DISCUSSION

Increasing levels of ROS after exposure to H_2O_2 have potentially toxic effects on human melanocytes (Mastore *et al.*, 2005). The inability of melanocytes to deal with oxidative stress has been implicated in vitiligo (Giovannelli *et al.*, 2004), and the Nrf2-ARE pathway plays an important function in the defense against oxidative stress. Therefore, the Nrf2-ARE pathway and its downstream genes may protect human melanocytes from H_2O_2-induced oxidative damage in vitiligo. In this study, we demonstrated that Nrf2/HO-1 signaling was directly involved in PIG1 cells and primary human melanocytes, and we established that HO-1 is a key factor in protecting melanocytes against H_2O_2-induced cell death. Furthermore, we proved that the induction of HO-1 by activating the Nrf2-ARE pathway protected melanocytes against H_2O_2-induced toxicity and that upregulation of Nrf2 expression diminished the H_2O_2-induced apoptosis of human melanocytes. Our study also suggested that the activation of H_2O_2-induced HO-1 transcription was at least partially mediated by the Nrf2-ARE pathway.

Compared with other ROS, H_2O_2 is quite stable and freely diffusible within and between cells (Pi *et al.*, 2003). H_2O_2 can oxidize DNA, proteins, or lipids, thus leading to the formation of many oxidized products that have been implicated in the onset of vitiligo (Lee and Shacter, 2000; Barbouti *et al.*, 2002; Trouba *et al.*, 2002). We found that human melanocytes treated with various concentrations of H_2O_2 underwent cell death in a concentration-dependent manner (Supplementary Figure S1 online), which was consistent with our previous report (Zhou *et al.*, 2009). Moreover, we further demonstrated that H_2O_2-induced cell death occurred by both apoptosis and necrosis, but we found that H_2O_2-induced cell death occurred mainly by apoptosis in PIG1 cells.

Nrf2 belongs to the CNC (cap 'n' collar) family of basic leucine zipper proteins (Kobayashi *et al.*, 1999), and Nrf2 regulates the expression of cytoprotective genes including *HO-1*, *NQO-1*, *GCL*, and *glutathione-S-transferases* (Itoh *et al.*, 1997; Ishii *et al.*, 2000). Activation of Nrf2 in cells provides an indirect way to enhance antioxidative capacity (Fahey and Talalay, 1999; Fahey *et al.*, 2002; Kensler *et al.*, 2007). Our study showed that increased expression of Nrf2 decreased H_2O_2-induced apoptotic cell death and that decreased expression of Nrf2 rendered melanocytes more vulnerable to H_2O_2-induced cell death. Our data are in accordance with reports in neurodegenerative disease research, which have shown that activation of Nrf2 by 1,2,4-triazine derivatives protects PC12 cells against apoptosis and that a kavalactone derivative protects against H_2O_2-induced PC12 cell death through Nrf2-ARE activation (Tanaka *et al.*, 2010; Tusi *et al.*, 2010). Because melanocytes and nerve cells have the same embryonic origin, it is possible that melanocytes have maintained these common characteristics.

Another interesting observation is the antioxidative role of HO-1. Nrf2 itself does not have any antioxidant functions. Previous studies have suggested that Nrf2 is inactivated in the cytoplasm and that oxidative stress activates Nrf2 by permitting translocation into the nucleus and then inducing the expression of its target antioxidant genes (Itoh *et al.*, 1999b; Nguyen *et al.*, 2003). Marrot *et al.* (2008) compared the modulation of phase II genes using three chemical inducers of Nrf2 pathway and solar UV or UVA in primary normal human melanocytes. These genes included *HO-1*, GCL subunits (*GCLC* and *GCLM*), and *NQO-1*, which appear to vary according to the inducers and cell types (Motohashi and Yamamoto, 2004). In this study, we found that these genes were upregulated in varying degrees after treatment with H_2O_2 in PIG1 cells. The largest increased expression was observed in HO-1 after treatment with H_2O_2 for 24 hours. Moreover, the role of HO-1 in this protective effect was further confirmed using ZnPP and hemin. Therefore, our finding suggested that the presence of HO-1 was crucial for melanocytes to cope with H_2O_2-induced oxidative stress. A similar antioxidant effect has also been reported in other

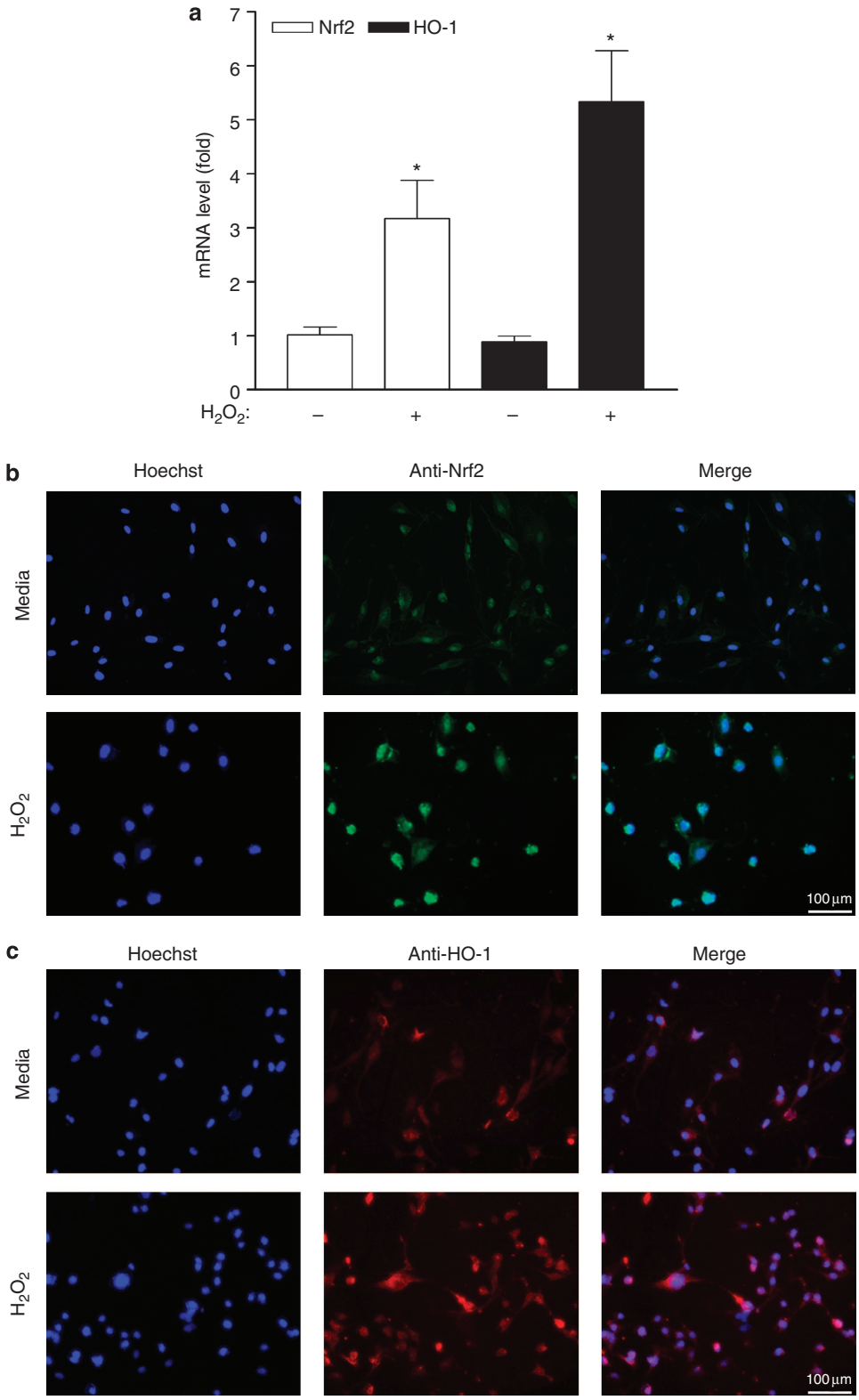

Figure 5. Modulation of the expression of nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in primary normal human melanocytes after treatment with 1.0 mM hydrogen peroxide (H_2O_2) for 24 hours. (**a**) The mRNA levels of Nrf2 and HO-1 were measured by real-time PCR (RT-PCR). Induction values represent the ratio of the gene expression levels in treated cells to those in untreated cells after normalization with the expression levels of the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene. $^{*}P<0.01$ compared with untreated cells. (**b**, **c**) The expression and nuclear translocation of Nrf2 and HO-1 were detected by immunocytochemistry. (**b**) Nrf2 was labeled with an antibody conjugated to FITC (green), and (**c**) HO-1 was labeled with an antibody conjugated to Cy3 (red). Hoechst 33258 was used to stain the nucleus (blue). Scale bar = 100 μm.

studies (Radhakrishnan *et al.*, 2005; Jeong *et al.*, 2006; Kim *et al.*, 2008; Lau *et al.*, 2008). Furthermore, many studies have shown that HO-1 and end products of HO-1 (biliverdin, carbon monoxide, and ferrous iron) have potent antioxidant and cytoprotective effects (Gopinathan *et al.*, 1994; Otterbein *et al.*, 2000; Ryter *et al.*, 2006; Lin *et al.*, 2007). Several studies have found that the action of the Nrf2-ARE/HO-1 pathway also has an important protective role in some cutaneous diseases. Beyer *et al.* (2007) demonstrated that Nrf2 regulates inflammation in skin wounds. Most importantly, this pathway can protect skin from carcinogenesis induced by chemicals. Moreover, Nrf2 and its downstream genes are involved in skin homeostasis in vitiligo patches (Natarajan *et al.*, 2010).

Two distinctly migrating forms of Nrf2 and HO-1 were detected by western blot analyses (Figures 1 and 4). The upper band of Nrf2 is a phosphorylated form, and the lower band of Nrf2 is a dephosphorylated form. The phosphorylated form of Nrf2 closely correlates with the nuclear localization of Nrf2 (Apopa *et al.*, 2008). Nrf2 translocates to the nucleus and then activates its target antioxidant genes, which have a major role in protecting the cell against oxidative damage (Itoh *et al.*, 1999a). The upper band of HO-1 is a native form (cytoplasmic form), and the lower band of HO-1 is a cleaved form (nuclear form). The nuclear and cytoplasmic HO-1 forms have equal roles in protecting cells against H_2O_2-mediated oxidative injury (Lin *et al.*, 2007). However, Lin *et al.* (2007) also suggested that nuclear HO-1 may enhance the protection against oxidative stress.

Several studies have reported that HO-1 expression is mainly regulated by Nrf2-dependent ARE activation in different cell types including the primary human melanocytes (Alam *et al.*, 2000; Ishii *et al.*, 2000; Marrot *et al.*, 2008). Our results suggest that the ARE-driven upregulation of HO-1 gene expression is mediated by activation of Nrf2 in PIG1cells. Nrf2 entering the nucleus and binding to the ARE in the HO-1 promoter region to trigger gene expression may be the predominant activation mechanism (Chen *et al.*, 2003). The augmentation of HO-1 may be accelerated in different cells by other means of transcriptional activation, such as activator protein-1 and signal transducers and activators of transcription. The involvement of these transcription factors in melanocytes will need to be elucidated in future studies.

In summary, this study clearly indicates that the Nrf2-ARE antioxidant pathway is one of the major cellular defense mechanisms, and that it is crucial for melanocytes to cope with oxidative stress. In addition, HO-1 can protect human melanocytes against H_2O_2-induced oxidative damage through an Nrf2-dependent pathway. Because oxidative stress has a critical role in the pathogenesis of vitiligo, we presume that any means to activate Nrf2 transcription or to enhance HO-1 levels may represent a valid approach to limit melanocyte damage in vitiligo lesions. Additional studies should be performed to identify the relationship between the Nrf2-ARE/HO-1 pathway and the pathogenesis of vitiligo. An *in vivo* study to further investigate our *in vitro* results may also be needed.

MATERIALS AND METHODS

Chemicals

ZnPP and hemin (Sigma, St Louis, MO) were prepared in a 10 mM stock solution of 100 mM sodium hydroxide (NaOH) (Thermo-Fisher Scientific, Loughborough, UK) and were further diluted in adequate cell culture medium. Analytical pure grade H_2O_2 was purchased from Xi'an Chemical Reagent Factory (Xi'an, China).

Cell culture

PIG1, an immortalized human epidermal melanocyte cell line (a gift from Dr Caroline Le Poole, Loyola University Chicago, Maywood, IL), and primary normal melanocytes that isolated from human foreskin specimens obtained during circumcision surgery were cultured in Medium 254 (Cascade Biologics/Invitrogen, Portland, OR) supplemented with human melanocyte growth supplement (Cascade Biologics/Invitrogen), 5% fetal bovine serum (Invitrogen, San Diego, CA), and a penicillin/streptomycin antibiotic mix (Invitrogen) at 37 °C in the presence of 5% CO_2. The PIG1 cell line was immortalized by retroviral introduction of the human papillomavirus type 16 E6 and E7 genes (Le *et al.*, 1997).

RT-PCR analysis

RT-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan) on a Chromo4 continuous fluorescence detector with a PTC-200 DNA Engine Cycler (Bio-Rad, Hercules, CA) to determine the relative mRNA levels of Nrf2, HO-1, NQO-1, GCLC, GCLM, and GAPDH (internal control). The cycling conditions were as follows: 95 °C for 2 minutes followed by 43 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C for 10 seconds, and extension at 72 °C for 15 seconds. The specificity of the PCR amplification was verified by a dissociation curve analysis. All reactions were run in triplicate. The primers used for RT-PCR are listed in Supplementary Table S1 online. Analyses of gene expression data were done using the $2_T^{-\Delta\Delta C}$ (Livak) method (Livak and Schmittgen, 2001).

Transient transfection of Nrf2

The pCMV6-XL5 mammalian expression plasmid containing human full-length Nrf2 was purchased from OriGene Technologies (Rockville, MD). PIG1 cells ($2–3 \times 10^5$ cells per well for 6-well plates and 8×10^3 cells per well for 96-well plates) were plated into each well and cultured for 24 hours. For overexpression of Nrf2, cells were transfected with pCMV6-XL5-Nrf2 (4 μg per well for 6-well plates and 0.2 μg per well for 96-well plates) at 90 to 95% confluency by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After a 6-hour incubation, cells were recovered in normal media after removal of transfection reagents and were then incubated for an additional 18 to 42 hours with or without drug treatment (H_2O_2, hemin, or ZnPP). Melanocytes in six-well plates were harvested 48 and 72 hours after transfection for assessing mRNA and protein changes, respectively. For the cells cultured in 96-well plates, the culture medium was switched to serum-free, antibiotic-free DMEM 48 hours after transfection, and the cells were then subjected to 1.0 mM H_2O_2 for 24 hours. The viability of pCMV6-XL5-Nrf2-transfected, pCMV6-XL5-transfected, and control melanocytes was then measured. The pCMV6-XL5 transfection (no Nrf2) and control cells (no transfection) were included in each experiment.

RNA interference

Three siRNAs specific for Nrf2 (Nrf2 siRNA1, 2, and 3) and scrambled sequence (scrambled siRNA) were designed and synthesized by Biomics Biotechnologies (Jiangsu, China). The siRNA sequences are listed in Supplementary Table S2 online. Melanocytes were transfected in antibiotic-free medium following the manufacturer's instructions. Briefly, healthy, growing, and adherent melanocytes were routinely trypsinized and resuspended to 1×10^5 cells ml^{-1} in normal growth medium without antibiotics, and the melanocytes were then set aside at 37 °C. After 24 hours, the cells were transfected with Nrf2 siRNA. Briefly, appropriate amounts of Nrf2 siRNA (100 pmol per well for 6-well plates and 5 pmol per well for 96-well plates) in serum-free DMEM/F12 medium (250 µl per well for 6-well plates and 25 µl per well for 96-well plates) and transfection reagent (Lipofectamine 2000; Invitrogen) in serum-free DMEM/F12 medium were prepared in separate centrifuge tubes (5 µl of transfection reagent in 245 µl of medium per well for 6-well plates and 0.25 µl of transfection reagent in 24.75 µl of medium per well for 96-well plates). After incubation for 5 minutes at 37 °C, the siRNAs and Lipofectamine were mixed, incubated at room temperature for an additional 20 minutes, and added to each well. The cell suspension that was previously set aside was transferred to the same wells containing the complex ($2–3 \times 10^5$ cells per well for 6-well plates and 8×10^3 cells per well for 96-well plates), and the cell suspension was mixed by gently rocking the plates back and forth. The medium was replaced by fresh medium 24 hours after transfection. The melanocytes in six-well plates were harvested 48 and 72 hours after transfection to assess mRNA and protein changes, respectively. At 48 hours after transfection, the culture medium for the melanocytes in 96-well plates was switched to serum-free, antibiotic-free DMEM, and the cells were then subjected to 1.0 mM H_2O_2 for 24 hours. The viability of Nrf2-silenced and control melanocytes was measured. Mock transfections (no siRNA) and scrambled transfections (using a nontargeting siRNA) were performed in each experiment. Three different Nrf2-specic siRNAs were tested, and Nrf2-siRNA3 was chosen to be used in further experiments because it was the most efficient and least cytotoxic among the siRNAs (Supplementary Figure S2 online).

Western blot analysis of Nrf2 and HO-1

After treatment, cells were washed two times with ice-cold phosphate-buffered saline (PBS) and were then lysed with a cell lysis solution (DSL, Webster, TX). Proteins were extracted from melanocytes and quantified with a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein were separated by 10% SDS-PAGE (Bio-Rad) and were then transferred from the gel to polyvinylidene difluoride membranes (Millipore, Billerica, MA). PageRuler Plus Prestained Protein Ladder (Fermentas, Hanover, MD) was used to confirm protein electrophoresis and transferring. After blocking in a solution of 5% non-fat dry milk diluted in Tris-buffered saline, the membranes were washed and then incubated with primary antibodies (rabbit polyclonal anti-Nrf2, 1:500; goat polyclonal anti-HO-1, 1:300; or mouse monoclonal anti-β-actin, 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:2,000; anti-goat IgG, 1:2,000; anti-mouse IgG, 1:5,000; Santa Cruz Biotechnology) for 2 hours at room temperature. Bound antibodies were detected using the ECL western blotting detection system (Millipore, Billerica, MA).

Annexin V-FITC/propidium iodide apoptosis assay

To quantify apoptotic and necrotic death, PIG1 cells were plated in six-well plates at a density of $2–3 \times 10^5$ cells per well overnight, and the cells were then transfected with pCMV6-XL5-Nrf2 and Nrf2-siRNA3. At 48 hours after transfection, the culture medium was switched to serum-free, antibiotic-free DMEM, and the cells were then exposed to 1.0 mM H_2O_2 for 24 hours. The cells were then collected and resuspended in 200 µl of binding buffer followed by the addition of 10 µl of FITC-labeled Annexin V and 10 µl of propidium iodide. After incubation in the dark (15 minutes at room temperature), the samples were diluted with 300 µl of binding buffer. Apoptotic and necrotic cells were identified using the Annexin V-FITC Apoptosis Detection Kit (MaiBio, Shanghai, China) following the manufacturer's instructions. The number of viable, apoptotic, and necrotic cells were quantified by flow cytometry (Beckman Coulter, Miami, FL), and the analysis was done with Expo32 software (Beckman Coulter). Each sample was repeated in triplicate.

Immunocytochemistry

Melanocytes were seeded in 24-well plates at a density of 1×10^4 cells per well. After the cells were cultured overnight, the medium was carefully removed, and the cells were then treated with 1.0 mM H_2O_2 in serum-free, antibiotic-free DMEM (Invitrogen) for an additional 24 hours. The cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were washed twice with PBS, preincubated in the same buffer containing 0.1% Triton X-100, and blocked with 0.1% BSA in PBS for 1 hour. The cells were incubated with primary antibodies (rabbit polyclonal anti-Nrf2, 1:50; and goat polyclonal anti-HO-1, 1:100; Santa Cruz Biotechnology) overnight at 4 °C. After three washes with PBS, cells were incubated with secondary antibodies (FITC-tagged goat anti-rabbit, 1:100; and Cy3-tagged rabbit anti-goat, 1:100; Cowin Biotech, Beijing, China) for 1 hour at room temperature. Cells were then washed in PBS and further incubated with 10 µg ml^{-1} Hoechst 33258 (Sigma) at room temperature for 10 minutes. Cells were observed under a fluorescent microscope equipped with a digital camera (Ti-s, Nikon, Japan). The images were captured using appropriate filters.

Statistical analysis

Each experiment was performed at least three times, and statistical analysis of the data was performed using unpaired two-tailed Student's *t*-test by GraphPad Prism (GraphPad Software 3.0; San Diego, CA). All data are expressed as mean ± SD. Values of $P < 0.05$ were considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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