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Vitiligo inducing phenols activate the unfolded protein response in melanocytes resulting in upregulation of IL6 and IL8

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Abstract

Vitiligo is characterized by depigmented skin patches due to loss of epidermal melanocytes. Oxidative stress may play a role in vitiligo onset, while autoimmunity contributes to disease progression. In this study we sought to identify mechanisms that link disease triggers and spreading of lesions. A hallmark of melanocytes at the periphery of vitiligo lesions is dilation of the endoplasmic reticulum (ER). We hypothesized that oxidative stress results in redox disruptions that extend to the ER, causing accumulation of misfolded peptides, which activates the unfolded protein response (UPR). We used 4-tertiary butyl phenol (4-TBP) and monobenzyl ether of hydroquinone (MBEH), known triggers of vitiligo. We show that expression of key UPR components, including the transcription factor X-box binding protein 1 (XBP1), are increased following exposure of melanocytes to phenols. XBP1 activation increases production of immune mediators interleukin-6 (IL6) and IL8. Co-treatment with XBP1 inhibitors reduced IL6 and IL8 production induced by phenols, while over-expression of XBP1 alone increased their expression. Thus, melanocytes themselves produce cytokines associated with activation of an immune response following exposure to chemical triggers of vitiligo. These results expand our understanding of the mechanisms underlying melanocyte loss in vitiligo and pathways linking environmental stressors and autoimmunity.

INTRODUCTION

Vitiligo vulgaris (“vitiligo”) is an acquired disorder characterized by depigmented skin patches due to localized loss of melanocytes. The etiology of vitiligo is not fully understood, but an intricate interaction of biochemical, environmental, genetic, and immunological factors contribute to its pathogenesis. A widely accepted model for the disease proposes that a trigger induces melanocyte stress that cannot be overcome in individuals susceptible to vitiligo. The initial stressors are not known in most cases (idiopathic vitiligo) but studies suggest that a common effect of the causative trauma is induction of oxidative stress (Boissy

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CONFLICTS OF INTEREST

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and Manga, 2004). Nonetheless, in certain patients, defined as having contact vitiligo, exposure to a chemical trigger such as 4-tertiary butyl phenol (4-TBP) and monobenzyl ether of hydroquinone (MBEH) is known to initiate the disease, which is subsequently indistinguishable from idiopathic vitiligo (Boissy and Manga, 2004). Individuals are primarily exposed to these chemicals in workplaces such as industries that manufacture phenols. One hypothesis proposes that these agents act as a substrate for tyrosinase, the rate limiting enzyme for melanogenesis, due to their structural similarity to tyrosine, which leads to the generation of reactive semi-quinone free radicals, induction of cellular stress (Boissy and Manga, 2004; Westerhof and d'Ischia, 2007) and cytotoxicity (Hariharan *et al.*, 2010).

Stressed melanocytes undergo apoptosis while also initiating targeting of melanocytes by the immune system. If the immune response is sustained, presumably determined by genetic factors, melanocyte death can be perpetuated, resulting in spread of lesions to areas distant from the initial site. Evidence for autoimmune-mediated disease progression in vitiligo includes its association with other autoimmune conditions such as type I diabetes; antibodies against melanocyte-specific antigens (Naughton *et al.*, 1983) in sera from patients; association between vitiligo susceptibility and polymorphisms at immune-related loci (Spritz, 2010); T-cell infiltration in margins of active lesions (Badri *et al.*, 1993; Le Poole *et al.*, 1996); and increased levels of multiple pro-inflammatory cytokines in sera and tissue from vitiligo patients (Kemp *et al.*, 2001; Moretti *et al.*, 2009; Tu *et al.*, 2003; Yu *et al.*, 1997).

Upon contact with melanocytes, 4-TBP and MBEH induce the production of reactive oxygen species (ROS) (Boissy and Manga, 2004; Manga *et al.*, 2006). We hypothesized that the resulting oxidative stress leads to disruption of homeostasis in the endoplasmic reticulum (ER) where oxidation/reduction reactions are critical for protein folding. Indeed, dilation of the melanocyte ER has been reported in biopsies from the edge of vitiligo lesions and in melanocytes cultured from vitiligo patients (Boissy *et al.*, 1991; Le Poole and Boissy, 1997; Le Poole *et al.*, 2000). Subsequent accumulation of misfolded peptides would then activate the unfolded protein response (UPR). The UPR, which consists of three pathways initiated by inositol-requiring enzyme-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6), respectively, promotes restoration of ER homeostasis and cell survival, but under sustained stress it can trigger apoptosis (Ron and Walter, 2007).

The UPR has been implicated in a number of autoimmune disorders associated with vitiligo such as type I diabetes (Lipson *et al.*, 2006). Similar to our proposed model for vitiligo, in diabetes, it has been suggested that exposure to environmental agents such as toxins may be involved in initiation of excessive ER stress in pancreatic β-cells, triggering an apoptotic cascade by the UPR that leads to autoimmunity (Fonseca *et al.*, 2009; Lipson *et al.*, 2006). Disruption of the UPR may contribute to development of autoimmunity through three possible mechanisms: 1) generation of antigens during degradation of misfolded proteins, 2) release of neo-antigens by apoptotic cells, and 3) disturbance of immune-tolerance mechanisms in cells with an abnormal UPR (Todd *et al.*, 2008).

Involvement of the UPR in the pathogenesis of vitiligo is suggested by genetic studies which found that polymorphisms in the gene encoding X-box binding protein 1 (*XBP1*), a transcription factor that modulates several downstream UPR targets, are associated with increased risk of developing the disease (Ren *et al.*, 2009). Activation of IRE1 by dimerization and phosphorylation induces splicing of mRNA encoding XBP1 (Merksamer and Papa, 2010).

The PERK-initiated UPR pathway reduces global protein synthesis via phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (EIF2α). Once homeostasis is restored, EIF2α is dephosphorylated by the growth arrest and DNA damage-inducible protein GADD34. PERK also activates the transcription factors ATF4 and nuclear factor erythroid 2-related factor 2 (NRF2) (Cullinan and Diehl, 2006). NRF2 regulates the expression of antioxidant responsive element containing genes including heme oxygenase-1 (*HMOX1*) (Nguyen *et al.*, 2009), which protects melanocytes from the deleterious effects of ultraviolet light as well as 4-TBP (Elassiuty *et al.*, 2011).

In this study we investigated the early events in induction of vitiligo by employing 4-TBP and MBEH, known triggers of vitiligo, to induce stress in human melanocytes. We show that exposure of melanocytes to these chemicals can activate the UPR and lead to enhancement of the antioxidant response, but also to increased expression of cytokines interleukin-6 (*IL6*) and *IL8* that may contribute to autoimmune-mediated progression of vitiligo. This study improves our understanding of the mechanisms that link environmental stressors and autoimmunity.

RESULTS

Doses for use in experiments were selected after the toxicities of 4-TBP and MBEH were determined, using a viability assay, 24 and 72 hours after exposure (Supplementary Figure S1 online). Concentrations of 250 μM and 300 μM were selected for dosing with 4-TBP and MBEH, respectively, because they resulted in less than 20% loss of viability. The rationale for selecting these doses was to stress cells but not to compromise their survival significantly, at least in early phases of exposure to phenols, in order to mimic the outcome of exposure to low concentrations of these agents in the environment.

There is evidence that, similar to the *in vitro* response of melanocytes to phenols, occupational vitiligo due to phenols is also dose dependent (James et al., 1977). Humans are exposed to various concentrations of phenols at work environment, however the precise doses are not known. The current Occupational Safety and Health Administration permissible exposure limit for phenol is 5 ppm. At these low concentrations, while phenols do not seem to induce immediate cell death, they act as enzyme inhibitors or may activate stress signaling pathways such as the unfolded protein response. Furthermore, we have previously shown that, at this dose, 4-TBP generates ROS in melanocytes (Manga et al., 2006).

4-TBP and MBEH induce upregulation of key UPR proteins

We used microarray analysis to investigate UPR-related changes in gene expression induced by 4-TBP and MBEH. Gene expression profiles of melanocytes dosed with 4-TBP or MBEH for 3, 6, and 24 hours were compared with expression in untreated cells. Of the 38,500 entities analyzed, 3960 were differentially expressed between treated and untreated cells (fold-change ≥ 3): of these, 176 genes were differentially expressed in both melanocytes dosed with 4-TBP and those dosed with MBEH (one way ANOVA, $p \leq 0.05$) after Benjamini-Hochberg correction for multiple comparisons. A total of 525 genes were differentially expressed only in cells dosed with 4-TBP and 3093 genes only in cells dosed with MBEH. Since both 4-TBP and MBEH are well known to induce vitiligo, we focused on changes in gene expression common to both agents.

Key UPR markers were found to be upregulated by exposure of melanocytes to both 4-TBP and MBEH. These include IRE1 and PERK; however the third UPR initiator, ATF6, was not increased in the presence of either phenol. These findings were validated using a UPR-focused quantitative PCR array (Supplementary Table S1 online). We also identified a number of changes in expression of genes that are not involved in the UPR, and are currently validating these findings.

Among the genes found to be increased by exposure to both 4-TBP and MBEH were *IL6* and *IL8*. Gene Ontology (GO) analysis based on gene function demonstrated 4-TBP and MBEH-induced upregulation of a number of cytokines and chemokines; genes involved in antigen processing and presentation such as intercellular adhesion molecule-1 (*ICAM1*), which may be involved in adhesion of leukocytes to melanocytes (Kirnbauer *et al.*, 1992); and genes reported in association with response to stressful stimuli in vitiligo such as superoxide dismutase-2 and *HMOX1* (Jian *et al.*, 2011; Yildirim *et al.*, 2004) (Supplementary Table S2 online). The most highly upregulated genes encoded cytokines and chemokines involved in immune responses. These findings suggest that 4-TBP and MBEH not only impact melanocyte viability, but may also contribute to inflammation/autoimmune-mediated progression of vitiligo.

4-TBP and MBEH induce an antioxidant response mediated by PERK and NRF2

PERK activation attenuates global protein synthesis via phosphorylation of EIF2α and an increase in ATF4 expression. Activation of this pathway mediates an antioxidant response through activation of the transcription factor NRF2. NRF2 is recruited to the nucleus where it regulates the expression of the antioxidant HMOX1. Levels of phospho-EIF2α have been shown to correlate with nuclear localization of NRF2 (Shiota *et al.*, 2010).

We have shown that exposure to 4-TBP or MBEH causes an increase in expression of PERK and its downstream target ATF4 (Figure 1a and Supplemental Table S1 online) as well as an increase in phosphorylation of EIF2α (Figure 1b). Sub-cellular fractionation and Western blot analysis demonstrate that melanocyte exposure to either 4-TBP or MBEH results in increased nuclear localization of NRF2 (Figure 1c) and mRNA expression of the antioxidant response regulator HMOX1 is increased (Figure 1d and e) compared with untreated cells, indicating that melanocytes mount an antioxidant response to both compounds.

Guanabenz binds to protein phosphatase 1, PPP1R15A/GADD34, disrupting dephosphorylation of EIF2α, and potentiating PERK signaling (Tsaytler *et al.*, 2011). Co-treatment of melanocytes with either 4-TBP or MBEH in combination with guanabenz resulted in increased HMOX1 (Figure 1d and e), supporting a role for PERK in the regulation of this key antioxidant enzyme.

Increased cytokine expression and secretion stimulated by vitiligo-inducing phenols

To validate our finding that the expression of certain cytokines, identified by microarray analysis, increase following exposure to vitiligo-inducing phenols, we performed quantitative RT-PCR array of 84 cytokines in cells treated with 4-TBP. Nineteen genes were upregulated significantly ($p < 0.05$) at one or more of the three time points of the study (Supplemental Table S3 online).

Results were confirmed using quantitative RT-PCR of individual mRNAs. Following 4-TBP treatment, IL6 and IL8 expression were significantly upregulated at 3 and 6 hours post treatment, while their expression was down-regulated 24 hours post treatment (Figure 2a), validating the microarray data.

4-TBP and MBEH induce production of IL6 and IL8 via the UPR

We performed Western blot analysis to investigate IRE expression and phosphorylation and semi-quantitative RT-PCR to assess XBP1 expression and splicing. Increased expression and phosphorylation of IRE1 by melanocytes was detected within 3 hours following 4-TBP or MBEH dosing (Figure 2b), concomitant with increased splicing of XBP1 (Figure 2c), leading to its expression, and indicating activation of the UPR following treatment with either 4-TBP or MBEH. Thus, 4-TBP and MBEH induce activation of the IRE1-XBP1 arm of the UPR.

IL6 and IL8 expression is regulated in part by XBP1 (Gargalovic *et al.*, 2006). Western blot analysis of proteins in the culture medium showed that within 3 hours of exposure to 4-TBP or MBEH, both IL6 and IL8 secretion by treated melanocytes was substantially higher than secretion by cells subjected to vehicle alone, thus correlating with activation of the IRE1 arm of the UPR (Figure 2d). Thapsigargin (TG), an inhibitor of sarco/endoplasmic reticulum calcium ATPases, and a well-known inducer of the UPR, was used as a positive control.

Two inhibitors of XBP1 splicing, salicylaldehyde (SA) (Volkmann *et al.*, 2011) and rapamycin, were used to block the IRE1 arm of the UPR in melanocytes prior to and concomitant with treatment with 4-TBP or MBEH. Although rapamycin is a well known inhibitor of the mammalian target of rapamycin (mTOR), it has also been shown to inhibit XBP1 splicing (Pfaffenbach *et al.*, 2010). Results of the RT-PCR experiments show that pre-treatment with both SA and rapamycin abrogated XBP1 splicing that follows exposure of melanocytes to 4-TBP or MBEH (Figure 3a). Quantitative PCR showed that when no inhibitor was added, 4-TBP and MBEH led to 9 fold and 9.4 fold increase in IL6 expression, respectively, compared to that seen in control cultures (Figure 3b). When XBP1 splicing was inhibited with SA, the levels of IL6 expression were markedly lowered to 0.9 fold and 2.2 fold, respectively, and to 0.9 fold and 1.3 fold expression, respectively, when rapamycin was

used. Likewise, treatment with SA reduced the induction of IL8 by 4-TBP and MBEH from 5.9 fold and 6.8 fold to 3.6 fold and 1.9 fold expression, respectively, and to 2.5 fold and 2.4 fold expression, respectively, when rapamycin was used.

To determine if XBP1 indeed mediated the increase in IL6 and IL8 expression, melanocytes were transfected with an expression vector containing the coding sequence of XBP1. Semi-quantitative RT-PCR showed that - similar to the effects observed with phenols - XBP1 overexpression was correlated with an increased expression of both IL6 and IL8 (Figure 3c). These results demonstrated that activation of XBP1, a key step in the UPR pathway, is involved in 4-TBP and MBEH-induced expression of IL6 and IL8 in melanocytes.

DISCUSSION

Oxidative stress and autoimmunity are key factors in the pathogenesis of vitiligo. In this study we demonstrate that stress response pathways activated by disruption of the cellular redox balance can induce both cellular antioxidant responses as well as expression of cytokines that may provoke an autoimmune-mediated progression of vitiligo.

A role for oxidative stress in vitiligo is supported by numerous studies. Antioxidant levels are elevated in sera from patients with vitiligo (Yildirim *et al.*, 2003), while cultured melanocytes from patients are more susceptible to oxidative stress (Manga *et al.*, 2006). We hypothesized that oxidative stress in melanocytes leads to disruption of the folding machinery of the ER, which is dependent upon redox reactions for formation of disulphide bonds. In support of this idea is ER dilation in melanocytes at the periphery of vitiligo lesions and in melanocytes cultured from vitiligo patients (Boissy *et al.*, 1991; Im *et al.*, 1994; Le Poole *et al.*, 2000). Accumulation of immature proteins in the ER results in activation of the UPR, a pathway also implicated in determining susceptibility to vitiligo in genetic association studies linking an XBP1 polymorphism with increased risk of developing the disorder (Ren *et al.*, 2009). Results of the current study provide evidence that the UPR is indeed activated by exposure of melanocytes to 4-TBP and MBEH, agents known to trigger vitiligo.

UPR activation is initially a cell survival mechanism. Initiation of PERK signaling leads to the recruitment of NRF2 to the nucleus and expression of the antioxidant enzyme HMOX1 (Cullinan and Diehl, 2004). We show that vitiligo-inducing phenols cause nuclear relocalization of NRF2 and HMOX1 expression. Sustained PERK signaling by inhibition of EIF2α dephosphorylation results in increased HMOX1 expression. Activation of the NRF2/HMOX1 pathway may play a role in reducing oxidative damage induced by phenolic compounds in human melanocytes. Activation of this pathway may be of particular importance in vitiligo as HMOX1 expression has been associated with suppression of dendritic cells that affect cytotoxic T-cell responses (Listopad *et al.*, 2007). Both cell types contribute to melanocyte killing in vitiligo lesions (Kroll *et al.*, 2005; van den Boorn *et al.*, 2009). Thus, PERK activation may reduce the toxicity of vitiligo-inducing phenols.

It has been suggested that 4-TBP and MBEH-induced melanocyte death results in activation of an autoimmune response in vitiligo, although 4-TBP has been shown to induce apoptosis,

while MBEH is thought to induce necrosis (Hariharan *et al.*, 2010). We therefore focused on those events that were common to cells treated with both 4-TBP and MBEH.

UPR signaling in endothelial cells has been shown to induce IL6 and IL8 expression (Gargalovic *et al.*, 2006). We found that both 4-TBP and MBEH caused increased production of IL6 and IL8. Pre-treatment of melanocytes with inhibitors of XBP1 activation resulted in decreased production of IL6 and IL8 following exposure to 4-TBP and MBEH. Moreover, transfection with an XBP1 vector was associated with increased expression of IL6 and IL8 similar to the effects observed with phenols. Thus, these vitiligo-inducing chemicals share in common the activation of a stress signaling pathway, which may be associated with an autoimmune response independent from these agents' direct chemotoxic effects.

The UPR may therefore serve as a key link between oxidative stress and production of pro-inflammatory cytokines, such as IL6 and IL8, that may promote autoimmune targeting of melanocytes. Increased IL6 has been found in sera of vitiligo patients (Tu *et al.*, 2003; Yu *et al.*, 1997) and lesional vitiligo tissues (Moretti *et al.*, 2009). IL6 is one of the key molecules that stimulate the immune reactions (Ishihara and Hirano, 2002) and stimulates T lymphocytes that induce autoimmune diseases (Gabay, 2006). Increased serum and/or tissue levels of IL6 have been documented in various autoimmune disorders associated with vitiligo (Alexandraki *et al.*, 2008; Kogiso *et al.*, 1992; Norose *et al.*, 1994; Salvi *et al.*, 1996). A molecular link between oxidative stress and autoimmune conditions such as age-related macular degeneration has been suggested by reports of increased production of IL6 from retinal melanocytes following stimulation with hydrogen peroxide (Wu *et al.*, 2010), supporting our hypothesis that UPR-mediated expression of IL6 links melanocyte stress and immune targeting of these cells. Moreover, IL6 significantly upregulates melanocyte ICAM1 expression (Kirnbauer *et al.*, 1992), which may facilitate leukocyte-melanocyte attachment and immunologic cytotoxicity. In the current study, 4-TBP and MBEH also induced expression of ICAM1 (Supplemental Table S2 online). Similarly, levels of IL8, a cytokine with prominent chemotactic activity, are elevated in sera of vitiligo patients (Yu *et al.*, 1997) and melanocytes produce IL8 following stimulation by anti-melanocyte IgG antibodies (Li *et al.*, 2000), which are present in most patients with vitiligo (Kemp *et al.*, 2001). IL8 may attract T-cells to vitiligo lesions leading to amplification of the inflammatory reaction and melanocyte death. Additionally, it has been shown that IL6 and IL8 may also directly inhibit the growth and modulate antigen expression of melanocytes (Krasagakis *et al.*, 1995).

A variety of skin cells including keratinocytes, endothelial cells, and fibroblasts as well as immune cells that are present in vitiligo tissue such as T-cells and dendritic cells are known to release IL6 and IL8. While each of these cells may contribute to the cytokine environment, dermatitis following exposure to vitiligo-inducing phenols depends on the presence of melanocytes in the skin (Nordlund *et al.*, 1985). Thus production of inflammatory cytokines from the melanocytes may be of particular relevance to the pathogenesis of vitiligo. Furthermore, early increases in expression of IL6 and IL8 following exposure to 4-TBP and MBEH, observed in our study, suggests a possible contributory role for these cytokines in the transition from chemical-induced melanocyte death to a persistent

autoimmune condition as observed in vitiligo. Our data demonstrate that in addition to IL6 and IL8, following treatment with phenols, melanocytes show increased expression of a variety of other immune response molecules. A number of these molecules have pro-inflammatory activities and may play a role in progression of vitiligo. In particular, IL11 is a pro-inflammatory cytokine that has IL6-like activity (Guk and Kuprash, 2011) and tumor necrosis factor (TNF) is found in tissues obtained from vitiligo patients (Birol *et al.*, 2006). Thus additional pathways contribute to the initiation of vitiligo. This is not surprising given the complex etiology of the disorder.

In summary, our findings support the hypothesis that activation of the ER-stress response plays a role in the pathogenesis of vitiligo and propose the following model: Vitiligo-inducing traumas cause oxidative stress in melanocytes leading to ER stress and activation of the UPR. Signaling via the UPR enhances the NRF2/HMOX1 antioxidant response and allows restoration of homeostasis. Individuals susceptible to vitiligo – for example due to their genetic background - are unable to sufficiently combat the oxidative stress, leading to sustained UPR activity, including IL6 and IL8 production. As a consequence, the persistent alterations in the immunological microenvironment of the skin, in combination with increased immunogenicity caused by phenols (van den Boorn *et al.*, 2011), may contribute to enhancement and expansion of vitiligo lesions. Thus the UPR may play a critical role in determining melanocyte viability following exposure to a vitiligo-inducing stressor and in the progression to an immune response that targets the melanocyte.

MATERIALS AND METHODS

Cell cultures and viability assay

Two lines of human melanocytes derived from normal neonatal skin (unrelated individuals) were obtained from Lifeline Cell Technology (Walkersville, MD) and cultured in Dermalife M melanocyte culture medium from the same company. Cells were treated with 1–1000 μM 4-TBP or MBEH for 24 and 72 h and a CellTiter96 assay (Promega, CA) was performed to assess cellular viability. Melanocytes were also treated with 150 nM thapsigargin (TG), which was used where noted as a positive control for induction of ER-stress response.

Microarray analysis

Individual total RNA samples from human melanocytes dosed with 4-TBP or MBEH for 3, 6, and 24 hours, as well as control cells before treatment were hybridized to Affymetrix Gene Chip Human Genome U133 Plus.2 (Agilent Technologies, Inc. Santa Clara, CA). Hybridization and processing was performed by the Genomics, Microarray Core Facility, New York University School of Medicine, NY. We analyzed the results using the Genespring GX 11.0 software (Agilent Technologies, Inc. CA). Data have been deposited in the NCBI microarray database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31641).

Quantitative real-time PCR

Total RNA was isolated from cell lysates using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was generated using the RT^2 First Strand Kit (SABiosciences Co, CA). Two RT^2

Profiler PCR Arrays were used to examine mRNA levels of human cytokines and UPR genes (SABiosciences Co, Frederick, MD). Real-time PCR was performed on an Applied Biosystems 7900 HT machine with SYBR Green RT^2/ROX PCR Master Mix solution (SABiosciences Co) following the manufacturers recommended PCR conditions. The average threshold cycle for each gene was determined from four independent experiments from each cell line. Values were exported to a template Excel file for analysis provided by the manufacturer.

Quantitative real-time PCR of IL6 and IL8 for the activation analysis with UPR inhibitors was performed using MyiQ™2 system (Bio-Rad Laboratories, Hercules, CA). SYBR Green RT^2/ROX PCR Master Mix solution (SABiosciences Co) was used for PCR reaction and forward and reverse primers were purchased from Sigma-Aldrich Co (Woodlands, TX). Data were obtained as Ct values and analyses were performed by the manufacturer's software. All reactions were performed in triplicate and the results are expressed as the mean of values from 2 separate experiments.

Western blot analysis

For protein extraction, cells were harvested in a Tris-base extraction buffer containing protease inhibitor. Fractionation of the cytoplasmic and nuclear NRF2 protein was performed using the NE-PER extraction reagents (Thermo Scientific Pierce, Rockford, IL) according to the manufacturer's instructions. Interleukins were detected in the supernatant media of cultured cells. Equal numbers of cells, 2×10^6 per sample, were cultured in 5 mL media that was subsequently collected and after brief centrifugation was concentrated using filtered centrifuge columns. After normalization with the total amount of protein in cell lysates, equal amounts of protein (for IL6 and IL8 detection) were loaded to each well for Western blot analysis. Protein concentrations were measured with bicinchoninic acid (BCA) protein assay. Twenty to forty micrograms of total protein were loaded and separated on 10–12% SDS-PAGE gels and then transferred to nitrocellulose membrane (Polyscreen; Perkin-Elmer, Waltham, MA). After blocking with 5% nonfat milk in TBS and 0.1% Tween 20, membranes were incubated overnight at 4° C with the following primary antibodies: mouse monoclonal antibodies for IL6 (1:500) and IL8 (1:500) (Santa Cruz Biotechnology, CA), IRE1 (1:1000) and EIF2α (1:5000) and rabbit monoclonal phospho-EIF2α (1:1000) (Cell Signaling, Danvers, MA), NRF2 (1:2000) (R&D Systems, Inc. Minneapolis, MN), and Rabbit polyclonal phospho-IRE1 (1:1000) (Novus Biologicals, Littleton, CO) and phospho-EIF2α (Cell Signaling, Danvers, MA). After washing, membranes were incubated for one hour at room temperature with horseradish peroxidase conjugated secondary antibodies. The immunoblots were developed using the enhanced chemoluminescence detection system (Amersham, Arlington Heights, IL). Actin (detected with mouse monoclonal anti-actin (1:10,000) (Sigma-Aldrich Co, St. Louis, MO)) and histone H1 (detected with goat anti-histone H1 (1:1000) (Santa Cruz)) were used as loading controls.

Reverse transcription-PCR

XBP1 splicing and IL6, IL8, and HMOX1 transcript expression were assessed by semi-quantitative RT-PCR. Cells were lysed and total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA). Following quantification, 1 μg of RNA was used to generate cDNA

using ImProm-II Reverse Transcription System (Promega, Madison, WI). RT-PCR was performed using the Gotaq assay kit in a Bio-Rad MJ mini personal thermal cycler PCR System (Life Science Research, CA). To differentiate the unspliced form of XBP1 from the spliced form, PCR products were incubated with Pst1 enzyme (New England BioLabs, Inc.). The PCR products were separated on a 2 % agarose gel and visualized by ethidium bromide staining. The unspliced XBP1 produced two 290-bp and 183-bp fragments while the spliced Xbp-1 generated a 447-bp fragment. IL6, IL8, and HMOX1 produced 223bp, 335bp, and 157bp bands, respectively. As the loading control, GAPDH and RPL13A were used.

Analyses using modulators of the UPR

On the basis of the findings in preliminary RT-PCR and Western blot studies, exposures in the presence of chemical inhibitors of XBP1 splicing were performed to confirm any potential role of UPR on any 4-TBP or MBEH-induced effects on human melanocytes. In these experiments, cells were exposed to 80 μM SA or 100 nM rapamycin 3 hours prior to exposure to 4-TBP or MBEH. The role of PERK pathway in production of IL6 and IL8 by human melanocytes was examined using guanabenz, a selective inhibitor of protein phosphatase 1 with protective effects against accumulation of misfolded proteins (Tsaytler *et al.*, 2011). Cells were pretreated with 50 μM guanabenz for one hour before being exposed to 4-TBP or MBEH.

Transient transfection with XBP1

A human XBP1 expression vector was purchased from the Arizona State University (Biodesign Institute, Tempe, AZ). The XBP1 cDNA was subcloned into the pcDNA3.2 expression vector (Invitrogen, Carlsbad, CA) and purified using the Qiagen Miniprep kit (Valencia, CA). DNA electroporation was performed using the Amaxa NHEM-Neo Kit (Lonza, Walkersville, MD). Each 0.5 million cells were transfected with 1 μg of purified expression vector or empty vector, as a control, according to the manufacturer's instructions. Dosing with 4-TBP was performed 48 hours after transfection and TG was used as a positive control for the UPR activation.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was assessed using a 2-sided *t*-test. $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATF6	activating transcription factor-6
EIF2α	eukaryotic initiation factor 2-alpha
ER	endoplasmic reticulum
GADD34	growth arrest and DNA damage-inducible protein
HMOX1	heme oxygenase-1
ICAM1	intercellular adhesion molecule-1
IL6	interleukin-6
IRE1	inositol-requiring enzyme-1
MBEH	monobenzyl ether of hydroquinone
NRF2	nuclear factor erythroid 2-related factor 2
PERK	protein kinase RNA (PKR)-like ER kinase
ROS	reactive oxygen species
4-TBP	4-tertiary butylphenol
UPR	unfolded protein response
XBP1	X-box binding protein-1

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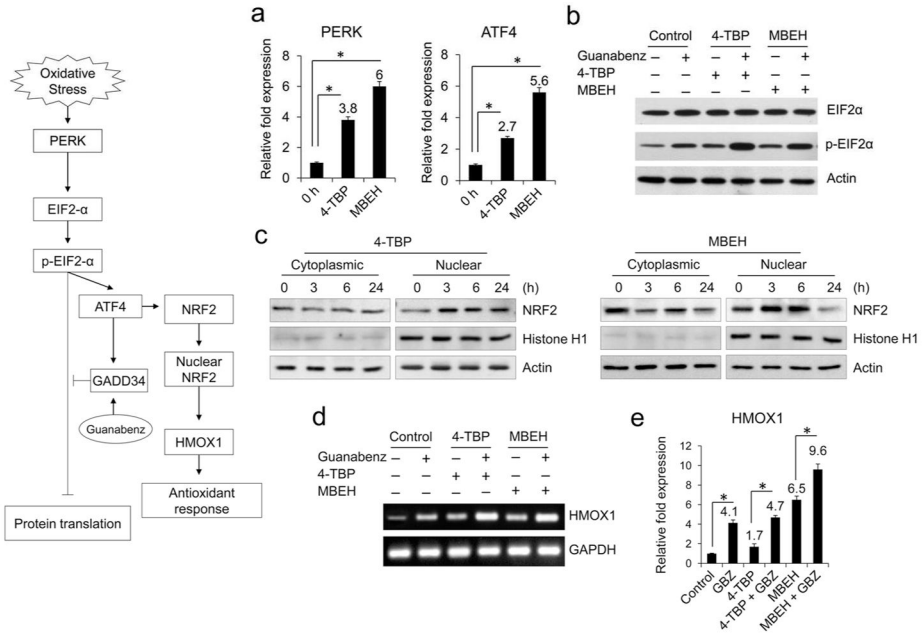

Figure 1. Treatment with 4-TBP or MBEH induces activation of the PERK and NRF2/HMOX1 pathways in human melanocytes

(a) Quantitative real-time PCR indicates that mRNA levels of PERK and ATF4 are increased in human melanocytes treated with exposure to either 4-TBP or MBEH versus controls (* $P < 0.01$ vs. 0 h). **(b)** Western blot analysis demonstrates increased levels of phosphorylated EIF2α in melanocytes following treatment with either 4-TBP or MBEH confirming UPR activation. **(c)** Western blot analysis of cytoplasmic and nuclear extracts of melanocytes shows a greater nuclear fraction of NRF2 following treatment with either 4-TBP or MBEH, **(d)** Both semi-quantitative RT-PCR and **(e)** quantitative real-time PCR demonstrate that mRNA levels of HMOX1 are increased following dosing with 4-TBP or MBEH and when dephosphorylation of EIF2α is inhibited with guanabenz, HMOX1 expression levels remain increased (* $P < 0.001$ vs. 0 h). GAPDH = loading control.

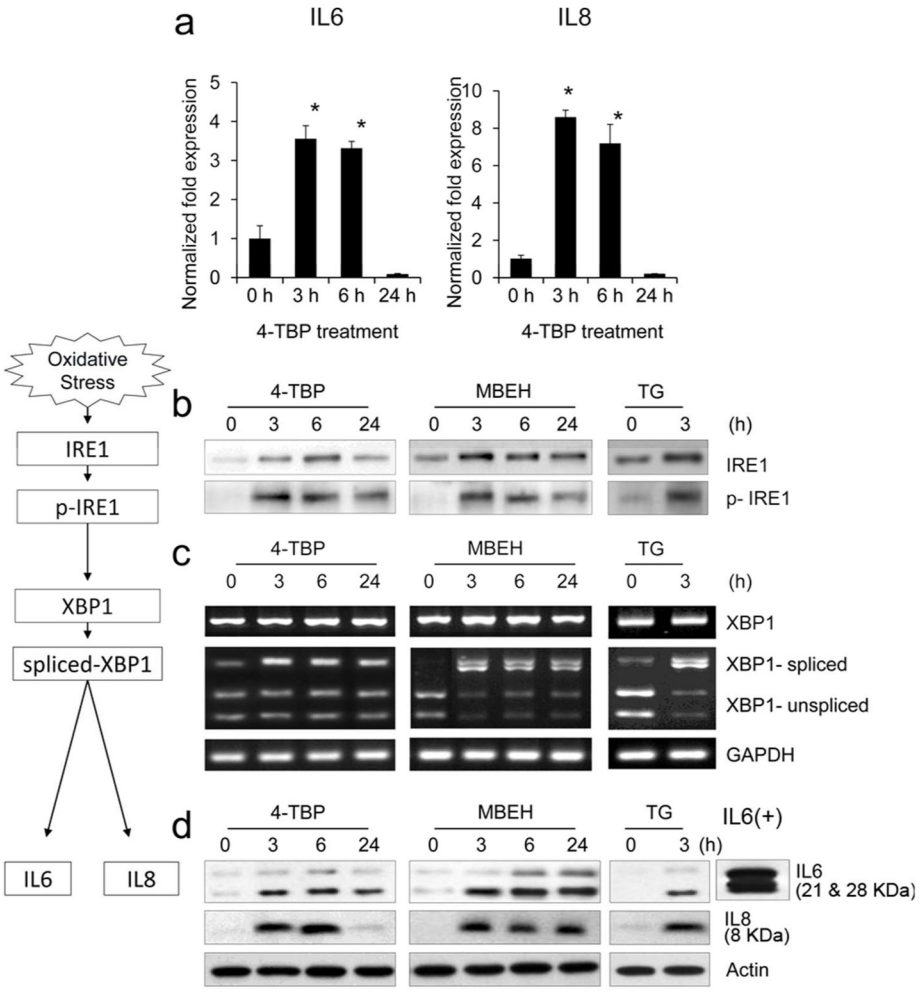

Figure 2. Treatment of melanocytes with 4-TBP or MBEH results in increased production of IL6 and IL8 by human melanocytes due to activation of the IRE1 initiated arm of the UPR

(a) Quantitative real-time PCR demonstrates that mRNA levels of IL6 and IL8 are increased in human melanocytes treated with 4-TBP versus controls (average of 4 experiments, two melanocyte lines, * $P < 0.001$ vs. 0 h). **(b)** Western blot analysis of cell lysates collected from human melanocytes treated with 4-TBP, MBEH, and thapsigargin (TG) showed increased expression of phosphorylated IRE1. **(c)** Semi-quantitative RT-PCR of RNA from human melanocytes treated with 4-TBP, MBEH, and TG showed increased splicing of XBP1 compared with control cells. **(d)** Western blotting of concentrated media collected from human melanocytes treated with 4-TBP or MBEH showed increased secretion of IL6 and IL8. Purified IL6 (Santa Cruz Biotechnology, Inc, CA) Cell Signaling, Danvers, MA), was used as a positive control. Three experiments with two different melanocyte lines yielded similar result. Three experiments performed with two different melanocyte lines, from unrelated donors, yielded similar results.

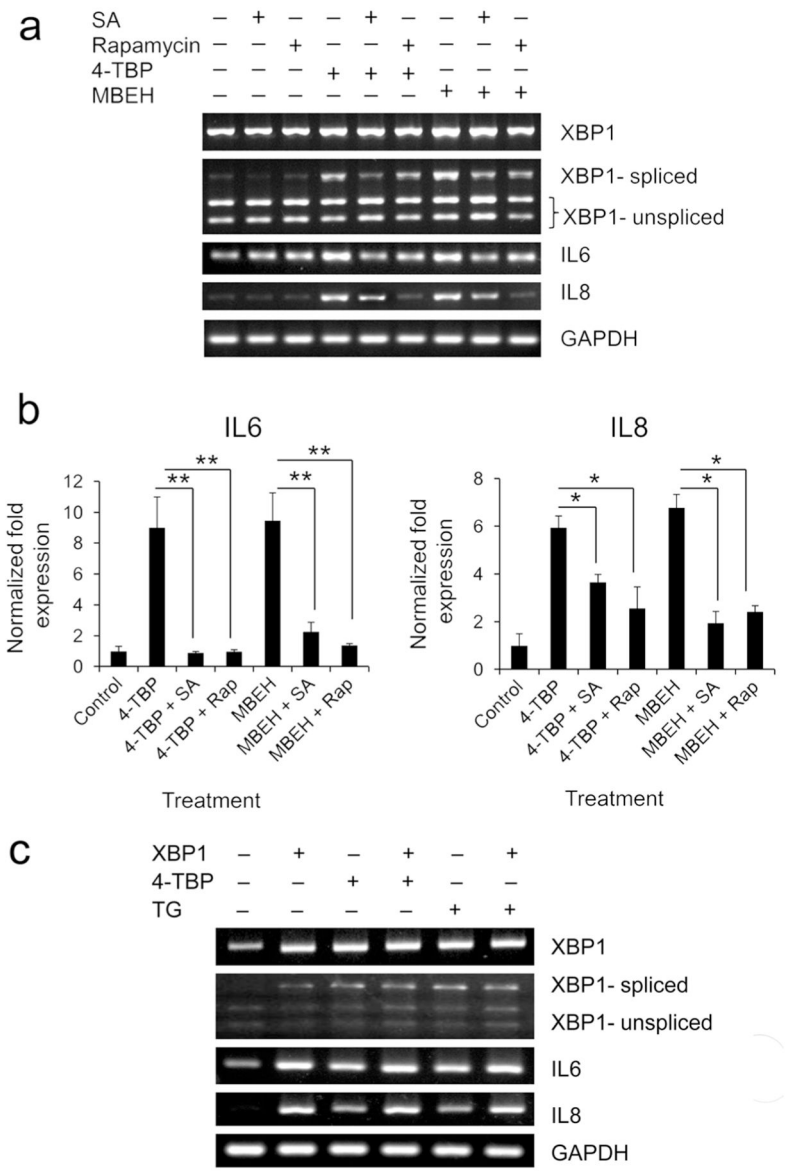

Figure 3. 4-TBP and MBEH induce production of IL6 and IL8 via the IRE1-XBP1 arm of the UPR

Melanocytes were pre-treated with salicylaldehyde (SA) and rapamycin, chemical inhibitors of XBP1 splicing, resulting in reduced production of IL6 and IL8 following treatment with 4-TBP and MBEH. **(a)** RT-PCR shows that pre-treatment with either SA or rapamycin can abrogate XBP1 splicing that follows exposure of melanocytes to 4-TBP or MBEH **(b)** Quantitative PCR shows that when XBP1 splicing was inhibited with SA or rapamycin, the levels of both IL6 and IL8 expression were lowered significantly (* $P < 0.001$, ** $P < 0.05$). Experiments performed with two different melanocyte lines were similar. **(c)** Semi-quantitative RT-PCR shows that transfection with an XBP1 vector results in increased expression and splicing of XBP1, and is correlated with an increase in IL6 and IL8 expression in melanocytes, similar to the effects of either 4-TBP or thapsigargin (TG).