Experimental dermatology • **Original article**

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Bilobalide protection of normal human melanocytes from hydrogen peroxide-induced oxidative damage via promotion of antioxidase expression and inhibition of endoplasmic reticulum stress

L. Lu, S. Wang, L. Fu, D. Liu, Y. Zhu and A. Xu

Third People's Hospital of Hangzhou, Hangzhou, China

doi:10.1111/ced.12664

Summary

Background. H_2O_2 accumulating in the epidermis contributes to the melanocyte damage characteristic of vitiligo. Removal of H_2O_2 by antioxidants is thus considered beneficial for patients with vitiligo.
Aim. To investigate the protective effects and underlying mechanism of bilobalide, the main terpenoid constituent of *Ginkgo biloba* extract, against H_2O_2-induced oxidative damage in normal human melanocytes.
Methods. Effects of bilobalide on melanocytes were investigated by measuring cell viability, heat shock protein (Hsp)70 release and levels of intracellular reactive oxygen species (ROS).
Results. Pretreatment of melanocytes with bilobalide for 24 h significantly inhibited H_2O_2-induced apoptosis, Hsp70 release and intracellular ROS increase in a dose-dependent manner. Bilobalide pretreatment increased the level of catalase (CAT) and glutathione peroxidase (GPx)1 mRNA in melanocytes. Although bilobalide moderately increased the phosphorylation of eukaryotic initiation factor 2, α subunit (eIF2α), suggesting its role in stimulating unfolded protein response signalling pathways, it also blocked the induction of anti-C/EBP homologous protein expression by H_2O_2.
Conclusion. This study indicates for the first time that bilobalide protects human melanocytes from oxidative damage by inhibiting H_2O_2-induced apoptosis and suppressing autoimmune response to melanocytes through reducing Hsp70 release. Instead of working as an ROS scavenger, bilobalide promotes the expression of antioxidases including CAT and GPx1, and inhibits H_2O_2-induced endoplasmic reticulum stress to protect melanocytes.

Introduction

Vitiligo is an acquired disease characterized by progressive loss of melanocytes. Its aetiology and pathogenesis are still unclear, hampering efforts to develop successful therapy. A number of mechanisms, including oxidative stress, melanocyte self-destruction, and autoimmune, neural and genetic factors have been proposed for the pathogenesis of vitiligo.[1] Although no single theory has proved sufficient to explain the pathogenesis of vitiigo, accumulating evidence indicates that oxidative stress plays an important role in the onset and progression of the disease.[2] The absence of melanocytes in patients with vitiligo indicates a close connection with redox imbalance in depigmented skin and peripheral blood cells.[3,4]

Hydrogen peroxide (H_2O_2), which is one of the main sources of reactive oxygen species (ROS), and has been found to accumulate in the epidermis of patients with acute vitiligo. Oxidative stress induced by H_2O_2 can

Correspondence: Dr Aie Xu, Department of Dermatology, 38 Xihu Blvd, Hangzhou 310009, China
E-mail: xuaiehz@msn.com

Conflict of interest: the authors declare that they have no conflicts of interest.

The first two authors contributed equally to this work, and should be considered joint first authors.

Accepted for publication 21 October 2014

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affect pigmentation by destroying epidermal pro-opiomelanocortin peptide redox homeostasis.[5] H_2O_2 is a major player in the regulation of cholinergic signalling, which is severely affected in the epidermis of patients with active vitiligo.[6] Furthermore, H_2O_2 is suggested to induce apoptosis directly in melanocytes, although the underlying mechanism for this is not clear. Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) triggers the unfolded protein response (UPR),[7] and H_2O_2-induced melanocyte apoptosis may have a strong connection with ER stress.[8] H_2O_2 is reported to promote the apoptosis of retinal pigment epithelial cells and HeLa cells by stimulating ER stress-related signalling pathways.[9]

Apart from the direct cytotoxic effects of H_2O_2 and other ROS on melanocytes, the role of oxidative stress in autoimmunity has been the subject of much research of late. High levels of H_2O_2 are believed to trigger increased turnover of surrogate substrates of tyrosinase, changing tyrosinase (which normally functions as an autoantigen) into a new antigen, leading to melanocyte-specific autoimmunity.[10] Moreover, vitiligo-inducing phenols have been shown to stimulate heat shock protein (Hsp)70 release from melanocytes to mediate the activation of dendritic cells (DCs) and stimulate the production of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 in melanocytes.[11]

Given this evidence, it is thus considered that removal of H_2O_2 by antioxidants is beneficial for the treatment of vitiligo.[5,6,10] *Ginkgo biloba* extract with defined antioxidant properties has been used clinically to treat vitiligo, and several clinical trials have confirmed that *G. biloba* extract can arrest the onset and progression of vitiligo.[12,13] Bilobalide is one of the main nonflavone components of *G. biloba* extract. It is a sesquiterpene trilactone, with a molecular weight of 326.3 Da, and has strong neuroprotective and antioxidant properties.[14] However, the ability of bilobalide to protect melanocytes from H_2O_2-induced oxidative damage is still unknown.

Therefore, the aim of the present study was to investigate whether bilobalide protects normal human melanocytes from H_2O_2-induced oxidative damage and to elucidate the underlying molecular mechanism of such protection.

Methods

Ethics statement

The study was approved by the institutional review board of the Third People's Hospital of Hangzhou, and written informed consent was obtained from each donor. All research was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Reagents

All culture medium and supplements used for the isolation and cultivation of melanocytes were obtained from Gibco (Carlsbad, CA, USA) with the exception of recombinant human basic fibroblast growth factor (bFGF; Pepro-Tech, Rocky Hill, NJ, USA), isobutylmethylxanthine (IBMX) and cholera toxin (CT) (both Sigma-Aldrich, St Louis, MO, USA). Antibodies against CCAAT/enhancer binding protein homologous protein (anti-CHOP), eukaryotic initiation factor 2, α subunit (eIF2α) and phosphorylated eIF2α (p-eIF2α) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Bilobalide and 30% H_2O_2 were obtained from the National Institutes for Food and Drug Control (Hangzhou, China).

Cell culture and treatment

Primary normal melanocytes isolated from human foreskin specimens obtained through circumcision surgery were cultured in Hu16 medium (F12 supplemented with 20 ng/mL bFGF, 20 μg/mL IBMX, 10 ng/mL CT, and 10% fetal bovine serum). Methods for the isolation and cultivation of melanocytes were as described previously.[15] Before bilobalide or H_2O_2 treatment, melanocytes were initially plated at a density of 1×10^4 cells per well in 96-well plates or at a density of 3×10^5 cells per well in 6-well plates, and incubated overnight. Bilobalide was prepared as a 100 mmol/L stock solution in DMSO, and DMSO without bilobalide was used for the negative control samples. H_2O_2 was freshly prepared from a 30% stock solution before each experiment.

Cell viability analysis

Cell viability was measured using a commercial kit [Non-Radioactive Cell Proliferation Assay (MTS); Promega, Madison, WI, USA] in accordance with the manufacturer's protocol. In brief, melanocytes were initially plated at a density of 1×10^4 cells per well in 96-well plates overnight, then cells were incubated with various concentrations of biolobalide (0–800 μmol/L) for 72 h to examine its cytotoxicity.

To investigate if bilobalide protects melanocytes from H_2O_2-induced cell damage, melanocytes were

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pretreated with bilobalide (0–400 μmol/L) for 1 h and then exposed to 1 mmol/L H_2O_2 for 16 h.

To measure cell viability, MTS solution was added to each culture well and incubated for 1 h at 37 °C. The absorbance at 490 nm was measured with a microplate spectrophotometer (SpectraMax190; MDC, Sunnyvale, CA USA), and the morphology of the cells examined under an inverted light microscope (IX70–141; Olympus, Tokyo, Japan).

Lactate dehydrogenase release assay

An LDH leakage assay was used to assess the cellular membrane integrity based on the amount of cytoplasmic LDH released into the medium. Melanocytes were initially plated into a 96-well plate. After pretreatment with different concentrations of bilobalide for 24 h, the cells were then exposed to H_2O_2 for 16 h. Supernatant from each well of the plate was transferred to the corresponding well of a 96-well enzymatic assay plate (Lactate Dehydrogenase Assay; Promega) and incubated with reconstituted substrate mix for 30 min. The reaction was stopped by the addition of stop solution, and the absorbance was measured with a microplate spectrophotometer at 490 nm.

Annexin V–propidium iodide staining

To determine whether bilobalide can attenuate H_2O_2-induced intracellular ROS elevation, melanocytes were pretreated with or without 25 or 100 μmol/L bilobalide for 24 h before being exposed to 1 mmol/L H_2O_2. Melanocyte apoptosis was detected by annexin V–propidium iodide (PI) staining assay, using a commercial kit (Annexin V-Propidium Iodide Apoptosis Detection Kit; Thermo Scientific, Carlsbad, CA, USA). Experimental melanocytes were detached with 0.05% trypsin–EDTA solution and resuspended in 100 μL of 1 × binding buffer containing fluorescein isothiocyante (FITC)-conjugated annexin V and PI, and incubated for 15 min at room temperature in the dark, then, 400 μL of 1 × binding buffer were added. Cells were analysed immediately with a flow cytometer (BD FACSCalibur; BD Biosciences, San Jose, CA, USA) to read annexin V-FITC at an excitation wavelength of 488 nm and emission wavelength of 530 nm, and to read PI at an excitation wavelength of 536 nm and emission wavelength of 617 nm. In total, 20 000 events from each sample were evaluated. CellQuest software (v5.1; BD Biosciences) was used for acquisition and analysis of data (Mac OSX v10.3.5). Cells on the two righthand quadrants of the dot plots were considered as apoptotic cells, with annexin V+/PI− cells representing early-stage and annexin V+/PI+ cells representing late-stage non-viable apoptotic cells.

Measurement of heat shock protein 70 release

Released extracellular Hsp70 was monitored using a commercial enzymatic immunoassay (EIA) kit (Hsp70 High Sensitivity EIA Kit; Enzo, Lausen, Switzerland) in accordance with the manufacturer's protocol. Briefly, 100 μL of Hsp70 standard or the experimental supernatant were transferred by micropipette into Hsp70 clear microtitre plates, and incubated for 2 h at room temperature. After removal of the samples, each plate was washed and then incubated with a polyclonal antibody specific for Hsp70, followed by a secondary antibody conjugated to horseradish peroxidase. A solution of tetramethylbenzidine and H_2O_2 was used as substrate, and the reaction was terminated by the addition of 1 N hydrochloric acid. Optical density was read at 450 nm on a microplate spectrophotometer. Hsp70 concentration of each sample was converted from standard curve.

Measurement of intracellular reactive oxygen species

Melanocytes were washed with PBS and incubated at 37 °C for 30 min with 2 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, Grand Island, NY, USA) diluted in serum-free medium, followed by 1 mmol/L H_2O_2 for 30 min. Cells were then detached and suspended in PBS. The intracellular ROS level was immediately analysed by by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm; 20 000 events from each sample were evaluated. CellQuest software was used to calculate average 2′,7′-dichlorofluorescein (DCF) fluorescence intensity from each cell measured.

RNA isolation and real-time reverse transcription PCR analysis

Total RNA was extracted from melanocytes (SV Total RNA Purification Kit; Promega), and reverse transcription (RT) performed in accordance with the manufacturer's instruction. Expression levels of CAT and GPx1 were measured using real-time RT-PCR analysis (Reverse Transcription Kit; Promega), and β-actin was used as internal control for normalization. Primers are shown in Table 1.

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Table 1 Primers used for real-time reverse transcription PCR analysis.

Primer	Direction	Sequence 5′→3′
CAT	Forward	TGGAGCTGGTAACCCAGTAGG
	Reverse	CCTTTGCCTTGGAGTATTTGGTA
GPx1	Forward	CAGTCGGTGTATGCCTTCTCG
	Reverse	GAGGGACGCCACATTCTCG
β-actin	Forward	ATAGCACAGCCTGGATAGCAACGTAC
	Reverse	CACCTTCTACAATGAGCTGCGTGTG

CAT, catalase; Gpx1, glutathione peroxidase-1.

Immunoblotting

After treatment, the cells were washed once with PBS and then lysed by adding 1 × SDS sample buffer. The proteins were separated by 10% SDS-PAGE, followed by transfer to a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% nonfat dry milk in TBS-T: 10 mmol/L Tris-HCL, 150 mmol/L NaCl and 0.1% Tween 20, pH 7.5) for 1 h at room temperature. After that, the membrane was incubated with corresponding primary antibodies against CHOP, p-eIF2α, eIF2α and β-actin overnight at 4 °C with gentle agitation. The membrane was then washed three times with wash buffer and incubated with fluorescent dye-labelled secondary antibody for 1 h at room temperature in the dark. The density of protein bands was measured by an infrared imaging system (Odyssey; LI-COR, Lincoln, NE, USA).

Statistical analysis

Data were expressed as the mean ± SD. Date normality was checked with Kolmogorov–Smirnov test and a one-way analysis of variance (ANOVA) was performed for multiple group comparison, followed by Dunnett *post hoc* test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant. Each experiment was performed in triplicate and repeated at least three times.

Results

Bilobalide exerts a protective effect against hydrogen peroxide-induced cytotoxicity to melanocytes

Using the cell viability assay, we found that there were no marked changes in the viability of melanocytes treated with 0–400 μmol/L bilobalide for 72 h, and cell viability slightly decreased after treatment with 800 μmol/L bilobalide (data not shown).

When melanocytes were pretreated with bilobalide (0–400 μmol/L) for 1 h and then exposed to 1 mmol/L H_2O_2 for 16 h, H_2O_2 caused severe inhibition of cell viability, and no marked protective effect of bilobalide on melanocytes was seen (Fig. 1a). However, stable and significant protective effects of bilobalide on melanocytes emerged when the duration of bilobalide pretreatment was extended to 24 h in the MTS assay (Fig. 1b).

We therefore chose two concentrations (25 and 100 μmol/L) of bilobalide and 24 h of pretreatment for the rest of the study. The morphological observations using inverted light microscopy indicated that exposure of melanocytes to H_2O_2 for 16 h resulted in obvious cell shrinkage and detachment, whereas pretreatment with 25 or 100 μmol/L bilobalide for 24 h significantly mitigated such morphological changes (Fig. 1c).

The LDH assay showed that extracellular LDH level increased along with the increase in number of dead cells. LDH leakage of melanocytes exposed to H_2O_2 increased about 3.5-fold compared with that of the untreated cells, suggesting that 1 mmol/L H_2O_2 is toxic to melanocytes. By contrast, pretreatment of melanocytes with bilobalide suppressed H_2O_2-induced LDH leakage in a concentration-dependent manner (Fig. 1e). This finding was in accordance with the results of the cell viability assay (Fig. 1d).

Bilobalide attenuates hydrogen peroxide-induced apoptosis in melanocytes

To investigate if bilobalide protects against H_2O_2-induced apoptosis, we used annexin V-PI assay. Figure 2b shows average apoptosis rates of three independent experiments. After exposure to 1 mmol/L H_2O_2 for 16 h, the average cell apoptosis rate increased from 12.63 ± 1.53% to 62.69 ± 6.08% ($P < 0.001$). After pretreatment with 25 or 100 μmol/L bilobalide, the H_2O_2-induced cell apoptosis rate reduced to 36.77 ± 4.75% and 25.80 ± 6.33%, respectively ($P < 0.001$), suggesting that bilobalide significantly attenuated H_2O_2-induced melanocyte apoptosis in a dose-dependent manner. Meanwhile, bilobalide caused no statistical change in basal apoptosis rate. Typical results are shown in Fig. 2a.

Bilobalide inhibits hydrogen peroxide-induced heat shock protein 70 secretion from melanocytes

Hsp70 secretion by melanocytes into the medium increased up to 2.5-fold in the presence of H_2O_2

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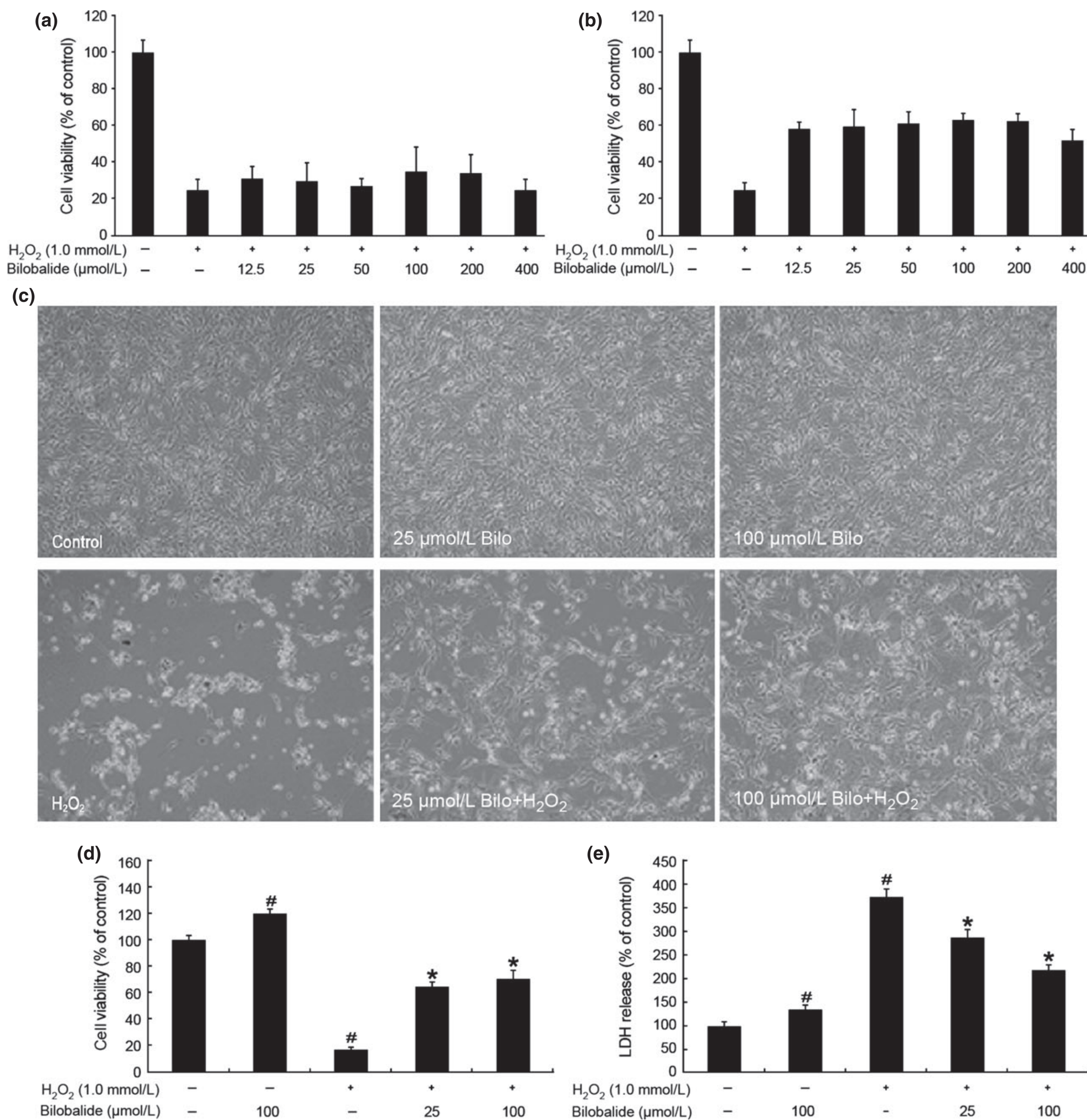

Figure 1 Protective effect of bilobalide on hydrogen peroxide (H_2O_2)-induced cytotoxicity to melanocytes. (a,b) Viability of melanocytes pretreated with 0–400 μmol/L of bilobalide for (a) 1 h and (b) 24 h, and then exposed to 1 mmol/L hydrogen peroxide (H_2O_2) for 16 h. (c) Effect of bilobalide on H_2O_2-induced morphological changes in melanocytes. (d,e) Protection of melanocyte viability by bilobalide was determined by (d) MTS and (e) lactate dehydrogenase (LDH) release assays. #$P < 0.05$ vs. untreated cells and *$P < 0.05$ vs. H_2O_2-treated cells. Bilo, bilobalide.

(Fig. 3) Although 100 μmol/L bilobalide alone induced a slight increase in Hsp70 secretion, bilobalide pretreatment markedly inhibited 1 mmol/L H_2O_2-induced Hsp70 secretion in a dose-dependent manner (25 μmol/L bilobalide $P = 0.002$; 100 μmol/L $P < 0.001$).

Bilobalide attenuates elevation of hydrogen peroxide-induced intracellular reactive species by promoting catalase and glutathione peroxidase-1 expression

As shown in (Fig. 4a), H_2O_2 stimulation markedly increased intracellular ROS. Bilobalide alone did not

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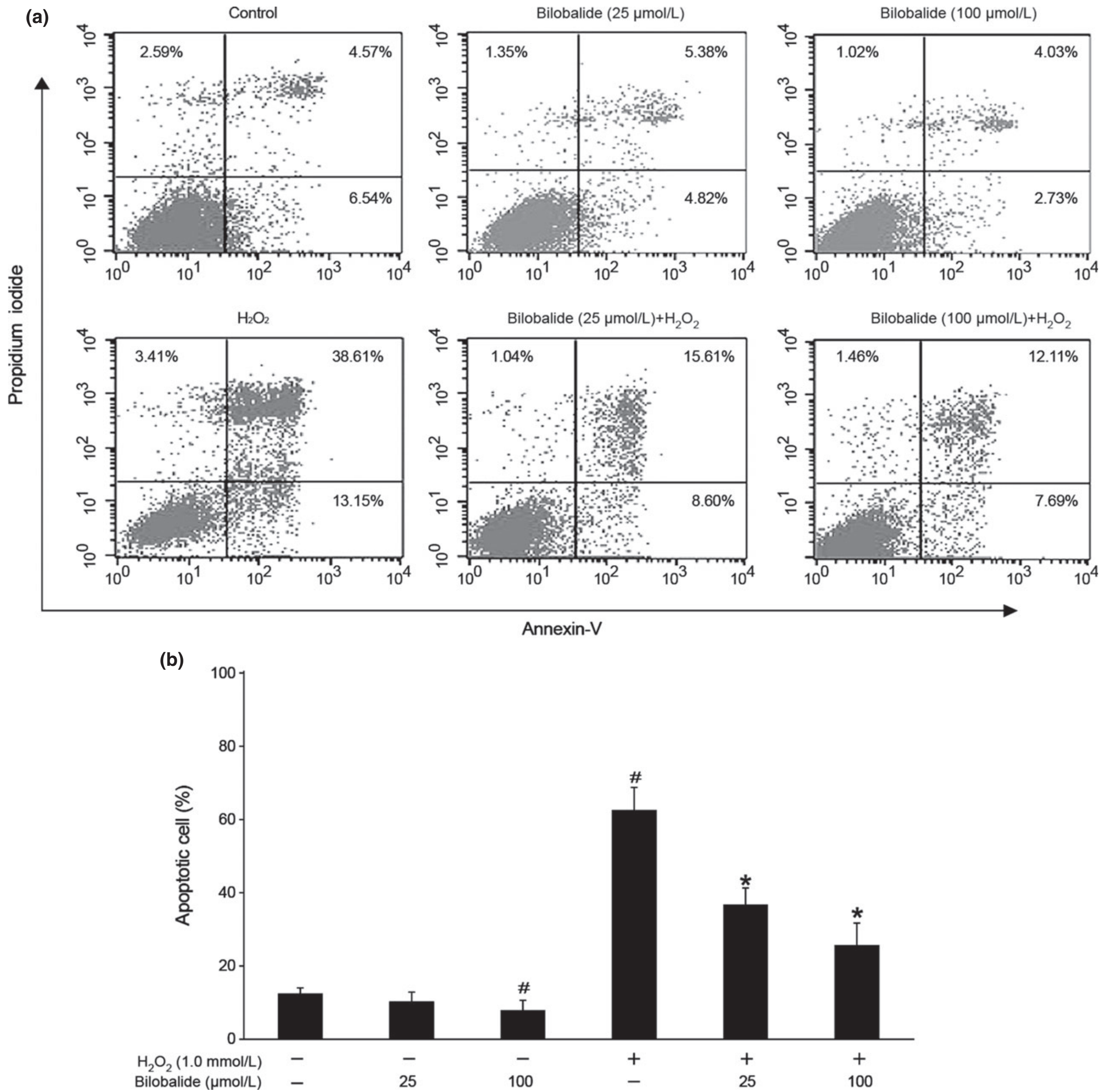

Figure 2 Inhibitory effect of bilobalide on hydrogen peroxide (H_2O_2)-induced apoptosis in melanocytes. Cells were pretreated with or without 25 or 100 μmol/L bilobalide for 24 h, and then incubated in the presence or absence of 1 mmol/L H_2O_2 for a further 16 h. Cellular apoptosis was assayed by fluorescein isothiocyante (FITC)-conjugated annexin V and propidum iodide (PI) counterstaining, and analysed by by flow cytometry. The representative flow cytometry figures were shown in (a), and the average apoptosis rates from three independent apoptosis experiments were shown in (b). #$P < 0.05$ vs. untreated cells and *$P < 0.05$ vs. cells treated with H_2O_2 alone.

significantly affect basal ROS level in melanocytes. However, pretreatment with 25 or 100 μmol/L bilobalide markedly suppressed the H_2O_2-induced increase in intracellular ROS_2.

As CAT and GPx1 (are the major intracellular antioxidases that remove $H_2O_{2.}$ we analysed the effect of bilobalide on CAT and GPx1 expression. With quantitative real-time RT-PCR analysis, we demonstrated that bilobalide markedly increased the mRNA expression of CAT (twofold at 25 μmol/L and 3.1-fold at 100 μmol/L, $P < 0.001$) and GPx1 (1.26-fold at 25 μmol/L, $P = 0.045$; 2.73-fold at 100 μmol/L, $P < 0.001$) compared with untreated cells, in a dose-dependent manner (Fig. 4b).

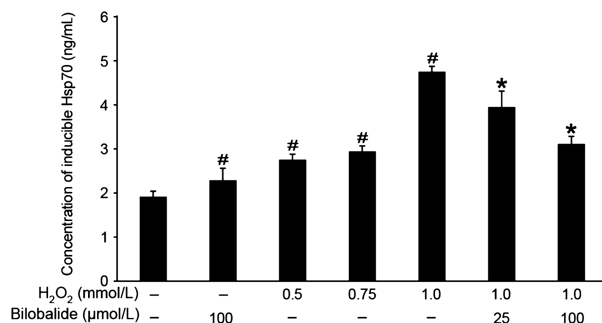

Figure 3 Decrease in hydrogen peroxide (H_2O_2)-induced Hsp70 secretion from melanocytes by bilobalide. Cells were pretreated with or without 25 or 100 μmol/L bilobalide for 24 h and then incubated in the presence or absence of H_2O_2 for a further 16 h. The concentration of secreted Hsp70 in the culture medium was measured by ELISA. #$P < 0.05$ vs. untreated cells; *$P < 0.05$ vs. cells treated with 1 mmol/L H_2O_2 alone.

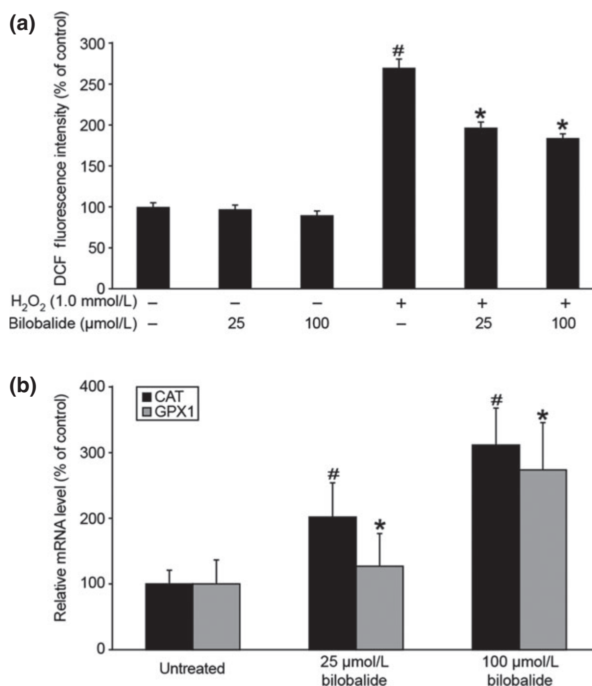

Figure 4 Effects of bilobalide on hydrogen peroxide (H_2O_2)-induced increase in intracellular reactive oxygen species (ROS). (a) Melanocytes were incubated in the presence or absence of 25 or 100 μmol/L bilobalide for 24 h, and then labelled with 2 μmol/L dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min, followed by incubation in the presence or absence of 1 mmol/L H_2O_2 for 30 min. After incubation, ROS levels were monitored by flow cyometry. (b) After pretreatment with or without bilobalide (25 or 100 μmol/L) for 24 h, expression of catalase (CAT) and glutathione peroxidase (GPx)1 mRNA was detected by quantitative reverse transcription (RT)-PCR. #$P < 0.05$ and *$P < 0.05$ for expression of CAT and GPx1, respectively, of bilobalide-treated cells vs. untreated cells.

Bilobalide stimulates unfolded protein response signalling and blocks hydrogen peroxide-induced C/EBP homologous protein expression

In order to investigate whether bilobalide has an inhibitory effect on H_2O_2-induced ER stress, we first examined the p-eIF2α level in melanocyte after treatment with bilobalide. Surprisingly, melanocyte exposure to 25 or 100 μmol/L bilobalide produced a slight increase in p-eIF2α suggesting that bilobalide itself can stimulate UPR signalling (Fig. 5a). p-eIF2α was strongly increased in melanocytes under H_2O_2 treatment at all time points indicating that ER stress was induced by H_2O_2. CHOP expression was induced 8 h after H_2O_2 was added (Fig. 5b). Pre-treatment of bilobalide did not change the p-eIF2α level. By contrast, expression of CHOP was blocked by bilobalide.

Discussion

Previous studies have suggested that *G. biloba* extract exerts its neuroprotectve effect through its free radical scavenger properties.[16] Bilobalide, the main constituent of the nonflavone fraction of *G. biloba* extract, has also been reported to have antioxidant and neuroprotective activities.[17] However, the functional properties of bilobalide have not been well characterized. In this study, we investigated the roles of bilobalide in protecting melanocytes against H_2O_2-induced oxidative damage, and explored the underlying mechanisms involved in this response. We found that bilobalide protects melanocytes against H_2O_2-induced apoptosis and Hsp70 release. In accordance with our results, bilobalide does not act simply as an H_2O_2 scavenger, as coculture of bilobalide with H_2O_2 was unable to alleviate H_2O_2 cytotoxicity to melanocytes. Bilobalide promotes the expression of two antioxidases, CAT and GPx1, in melanocytes. These are the major cellular enzymes involved in H_2O_2 removal, thus high levels of CAT and GPx1 help to reduce the oxidative damage to melanocytes caused by of H_2O_2. In addition, our study revealed that bilobalide pretreatment blocks the H_2O_2-induced upregulation of the apoptosis-related transcription factor CHOP [also known as growth arrest and DNA-damage-inducible protein (GADD153)]. Other studies have suggested that bilobalide may bind to various receptors in different cell types and it can activate the PI3K/AKT pathway to prevent apoptosis in SH-SY5Y cells.[18] Thus, the mechanism of melanocyte protection by bilobalide is complex and may involve multiple signalling pathways.

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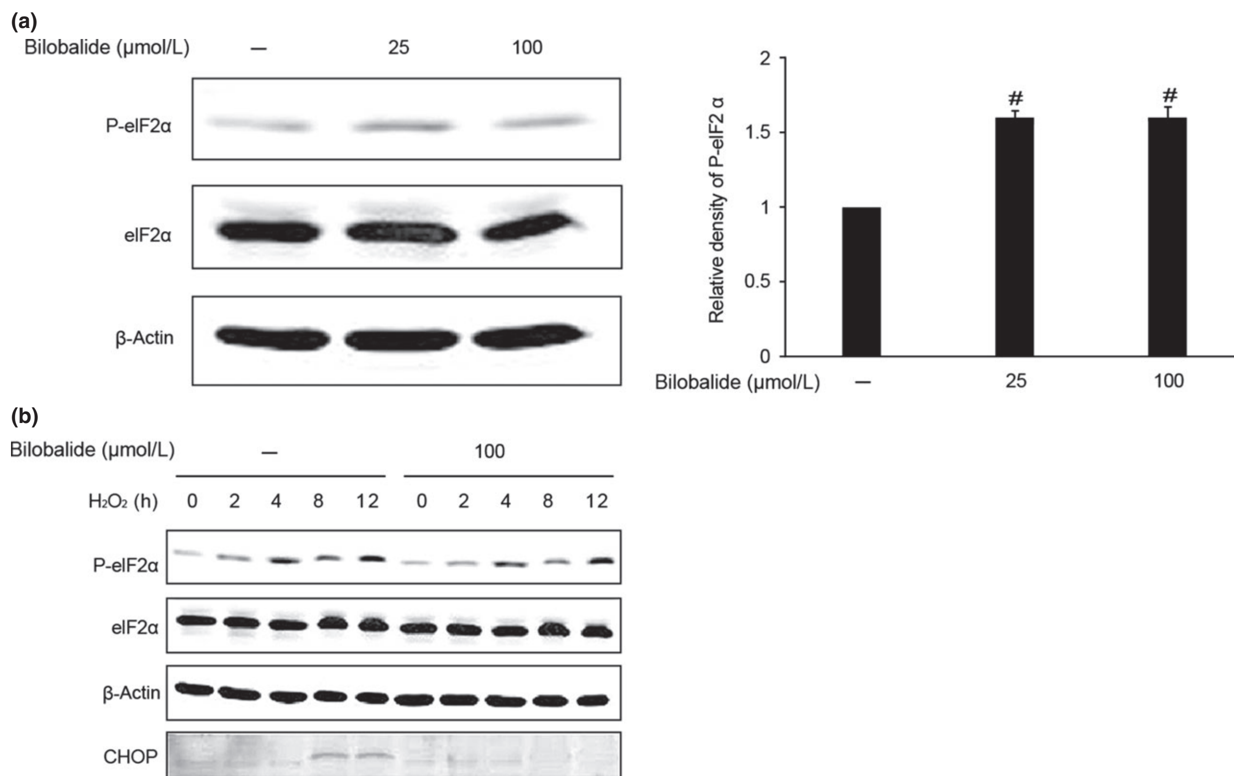

Figure 5 Upregulation of eukaryotic translation initiation factor 2, α subunit (eIF2α) phosphorylation and selective block of hydrogen peroxide (H_2O_2)-induced C/EBP homologous protein (CHOP) expression by bilobalide. (a) p-eIF2α in melanocytes treated with 25 or 100 μmol/L bilobalide for 24 h was assessed by western blotting (left) and the relative intensity of eIF2α phosphorylation bands was normalized to that of total eIF2α, and presented as mean ± SD from three experiments. #$P < 0.05$ vs. untreated cells. (b) Melanocytes were incubated with or without 100 μmol/L bilobalide for 24 h before treatment with 1 mmol/L H_2O_2 for the indicated times, and expression of phosphorylated (p)-eIF2α, eIF2α and CHOP was assessed by western blotting analysis. β-actin was used as an internal control.

Hsps are a group of molecular chaperones that are among the most highly conserved and immunogenic proteins across different species. Hsps have been implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis, atherosclerosis and type 1 diabetes.[19] Enhanced expression of Hsps under conditions of stress might trigger an autoimmune response by presenting a previously hidden antigen to the immune system. Hsps can also reinforce the immune response by activating DCs. We found in this study that various concentrations of H_2O_2 induced Hsp70 release from melanocytes. Barfuss *et al.* showed previously that overexpression of inducible Hsp70 (Hsp70i), together with a melanocyte-specific target antigen, tyrosinase-related protein-2 of human origin, could induce progressive depigmentation in wild-type C57BL/6 mice, but not in Hsp70i knockout mice.[20] Hsp70-mediated DC activation disrupts T-cell tolerance toward self antigens, and enhances self-reactive cytotoxic T-lymphocyte infiltration.[21] Furthermore, an Hsp70 variant with a mutation in its DC-activating region is capable of reversing the autoimmune depigmentation in vitiligo by binding to DCs and reducing their activation.[22] This cumulative evidence therefore indicates that enhancement of Hsp70 by H_2O_2 might contribute to induction of vitiligo by oxidative stress. Our results demonstrate that pretreatment of melanocytes with bilobalide markedly inhibits H_2O_2-induced Hsp70 release in a dose-dependent manner. Therefore, bilobalide plays dual roles in melanocyte protection under oxidative stress conditions through preventing the potential autoimmune response and inhibiting the direct killing of melanocytes. However, the mechanism of Hsp70 release inhibition by bilobalide needs to be elucidated further.

The ER is the location for folding and maturation of proteins before they are delivered to other cellular compartments.[23] The UPR is activated by accumulation

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of unfolded or misfolded proteins in the lumen of the ER. UPR signalling promotes restoration of ER homeostasis and cell survival, but under sustained ER stress it can trigger apoptosis.[24] The UPR consists of three pathways initiated by inositol-requiring enzyme (IRE)1, protein kinase RNA-like ER kinase (PERK) and activating transcription factor (ATF)6, respectively. The PERK-initiated UPR pathway leads to a transient inhibition in protein translation via phosphorylation of eIF2α. Meanwhile, activated IRE1 splices the mRNA of X-box binding protein (XBP)-1, transforming it into a fully active transcription factor, which regulates a number of downstream components of the UPR. Activation of IRE1, PERK and ATF6 in response to ER stress is thought to occur in parallel, but the activation of each one differs in timing and duration.[24] H_2O_2-induced melanocyte apoptosis may have a strong connection with ER stress. It has been reported that CHOP/GADD153 is an important component of the ER stress-mediated apoptosis pathway.[25] In our study, we found that 25 or 100 μmol/L bilobalide slightly increased phosphorylation of eIF2α, suggesting that prolonged bilobalide treatment stimulates UPR in melanocytes. Because bilobalide alone does not cause apoptosis in melanocytes, activation of UPR by bilobalide seems to 'prime' melanocytes for resistance to other stresses such as H_2O_2. It has been reported that XBP1 enhances the expression of CAT,[26] and we thus hypothesize that upregulation of CAT and GPx1 by bilobalide is also mediated by activated XBP1 following UPR.

eIF2α-dependent inhibition of translation leads to the induction of the transcription factor CHOP, which is often considered to a significant mediator of apoptosis under ER stress.[23] CHOP is induced by 1 mmol/L H_2O_2 at 8 and 12 h, suggesting that it mediates the apoptosis in melanocytes caused by H_2O_2. CHOP expression is primarily induced through the PERK–eIF2α–ATF4 pathway, although IRE1-XBP1 and ATF6 pathways also contribute.[27] In our study, pretreatment with 100 μmol/L bilobalide for 24 h blocked the induction of CHOP by H_2O_2 without changing the eIF2α phosphorylation pattern, indicating that bilobalide can selectively inhibit the apoptosis-related signalling branch of UPR signals to protect melanocytes.

Conclusion

Bilobalide may ameliorate H_2O_2-induced ER stress and apoptosis in human melanocytes by promoting CAT and GPx1 synthesis, and by blocking apoptosis-related CHOP induction, possibly through modulating UPR signalling. Our results partly explain the mechanism underlying the therapeutic efficacy of *G. biloba* extract in vitiligo treatment. Further studies should be performed to determine the biological efficacy and precise mechanisms of bilobalide *in vitro* and *in vivo*.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 81071294), the Science Foundation of Ministry of Health (Grant NO: WKJ2012–2–036), the Natural Science Foundation of Zhejiang, China (grant nos Z2100973 and LY13H110001), the Public Applied Technological Research Project of Zhejiang Province (grant no. 2011C23134), the Key Scientific Innovation Program of Hangzhou (grant no. 20122513A02), and the National Key Clinical Specialty Construction Project of China.

What's already known about this topic?

- Bilobalide, the main constituent of *G. biloba* extract, has been reported to have antioxidant and neuroprotective activities.
- *G. biloba* extract has been used clinically to treat vitiligo.

What does this study add?

- Bilobalide may ameliorate H_2O_2-induced ER stress and apoptosis in human melanocytes by promoting CAT and GPx1 synthesis and by blocking apoptosis-related CHOP induction, possibly through modulating UPR signalling.

References

1 Namazi MR. Neurogenic dysregulation, oxidative stress, autoimmunity, and melanocytorrhagy in vitiligo: can they be interconnected? *Pigment Cell Res* 2007; **20**: 360–3.

2 Maresca V, Roccella M, Roccella F *et al.* Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. *J Invest Dermatol* 1997; **109**: 310–13.

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3 Dell'Anna ML, Maresca V, Briganti S *et al.* Mitochondrial impairment in peripheral blood mononuclear cells during the active phase of vitiligo. *J Invest Dermatol* 2001; **117**: 908–13.

4 Schallreuter KU, Rübsam K, Gibbons NC *et al.* Methionine sulfoxide reductases A and B are deactivated by hydrogen peroxide (H_2O_2) in the epidermis of patients with vitiligo. *J Invest Dermatol* 2008; **128**: 808–15.

5 Spencer JD, Gibbons NC, Rokos H *et al.* Oxidative stress via hydrogen peroxide affects proopiomelanocortin peptides directly in the epidermis of patients with vitiligo. *J Invest Dermatol* 2007; **127**: 411–20.

6 Grando SA, Pittelkow MR, Schallreuter KU. Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. *J Invest Dermatol* 2006; **126**: 1948–65.

7 Manga P, Bis S, Knoll K *et al.* The unfolded protein response in melanocytes: activation in response to chemical stressors of the endoplasmic reticulum and tyrosinase misfolding. *Pigment Cell Melanoma Res* 2010; **23**: 627–34.

8 Tavender TJ, Bulleid NJ. Peroxiredoxin IV protects cells from oxidative stress by removing H_2O_2 produced during disulphide formation. *J Cell Sci* 2010; **123**: 2672–9.

9 Pallepati P, Averill-Bates DA. Activation of ER stress and apoptosis by hydrogen peroxide in HeLa cells: protective role of mild heat preconditioning at 40°C. *Biochim Biophys Acta* 2011; **1813**: 1987–99.

10 Laddha NC, Dwivedi M, Mansuri MS *et al.* Vitiligo: interplay between oxidative stress and immune system. *Exp Dermatol* 2013; **22**: 245–50.

11 Toosi S, Orlow SJ, Manga P. Vitiligo-inducing phenols activate the unfolded protein response in melanocytes resulting in upregulation of IL6 and IL8. *J Invest Dermatol* 2012; **132**: 2601–9.

12 Parsad D, Pandhi R, Juneja A. Effectiveness of oral Ginkgo biloba in treating limited, slowly spreading vitiligo. *Clin Exp Dermatol* 2003; **28**: 285–7.

13 Szczurko O, Shear N, Taddio A, Boon H. Ginkgo biloba for the treatment of vitilgo vulgaris: an open label pilot clinical trial. *BMC Complement Altern Med* 2011; **11**: 21.

14 Lang D, Kiewert C, Mdzinarishvili A *et al.* Neuroprotective effects of bilobalide are accompanied by a reduction of ischemia-induced glutamate release in vivo. *Brain Res* 2011; **1425**: 155–63.

15 Hong WS, Hu DN, Qian GP *et al.* Ratio of size of recipient and donor areas in treatment of vitiligo by autologous cultured melanocyte transplantation. *Br J Dermatol* 2011; **165**: 520–5.

16 Zhou LJ, Zhu XZ. Reactive oxygen species-induced apoptosis in PC12 cells and protective effect of bilobalide. *J Pharmacol Exp Ther* 2000; **293**: 982–8.

17 Bastianetto S, Zheng WH, Quirion R. The Ginkgo biloba extract (EGb 761) protects and rescues hippocampal cells against nitric oxide-induced toxicity: involvement of its flavonoid constituents and protein kinase C. *J Neurochem* 2000; **74**: 2268–77.

18 Shi C, Wu F, Yew DT, Xu J *et al.* Bilobalide prevents apoptosis through activation of the PI3K/Akt pathway in SH-SY5Y cells. *Apoptosis* 2010; **15**: 715–27.

19 Rajaiah R, Moudgil KD. Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev* 2009; **8**: 388–93.

20 Mosenson JA, Zloza A, Klarquist J *et al.* HSP70i is a critical component of the immune response leading to vitiligo. *Pigment Cell Melanoma Res* 2012; **25**: 88–98.

21 Denman CJ, McCracken J, Hariharan V *et al.* HSP70i accelerates depigmentation in a mouse model of autoimmune vitiligo. *J Invest Dermatol* 2008; **128**: 2041–8.

22 Mosenson JA, Zloza A, Nieland JD *et al.* Mutant HSP70 reverses autoimmune depigmentation in vitiligo. *Sci Transl Med* 2013; **5**: 174ra28.

23 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011; **334**: 1081–6.

24 Lin JH, Li H, Yasumura D *et al.* IRE1 signaling affects cell fate during the unfolded protein response. *Science* 2007; **318**: 944–9.

25 Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004; **11**: 381–9.

26 Liu Y, Adachi M, Zhao S *et al.* Preventing oxidative stress: a new role for XBP1. *Cell Death Differ* 2009; **16**: 847–57.

27 Song B, Scheuner D, Ron D *et al.* Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest* 2008; **118**: 3378–89.

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