

pass the blood–brain barrier. AD is a chronic disease that requires long-term drug treatment in order to produce therapeutic effects. Thus, this property of AE3-208 would be of great advantage for its clinical use. As for the mechanism underpinning the amelioration of cognitive dysfunction in the APP23 mice following the administration of AE3-208, we believe that this is mediated by a similar mechanism to EP₄ receptor deletion, given that oral administration of AE3-208 decreases levels of A β and γ -secretase activity and inhibits the localization of γ -secretase in endosomes. The soluble A β level was reduced in APPsw/EP₄^{-/-} mice but not in mice administered with AE3-208. This difference would be because of the difference in extent of the inhibition; deletion of the gene encoding EP₄ receptor completely inhibits the function of this protein, whereas administration of the drug may cause partial inhibition. As for the difference between soluble and insoluble A β for the modulation by administration of AE3-208, we have no clear explanation at present. One possible explanation is that the temporal alteration in synthesis of A β may affect more drastically soluble A β level than insoluble one.

Although this study focused on how inflammation affects the pathogenesis of AD through PGE₂ but not on how inflammation is induced in association with AD progression, we examined the effect of inhibition of EP₄ receptor on the activation of astrocytes. As shown in Fig. S3, the expression of glial fibrillary acidic protein (a marker for the activity of astrocytes) was higher in 18-month-old APPsw/EP₄^{+/+} mice than in WT/EP₄^{+/+} and APPsw/EP₄^{-/-} mice. As for AE3-208, because we used 6-month-old mice, the activation of astrocytes by the expression of APPsw was not so clear; however, the activity was a little lower in drug-treated mice than in control mice (Fig. S3). These results suggest that the inhibition of EP₄ receptor suppresses APPsw-mediated activation of astrocytes (inflammation). Based on previously reported results, the activation of EP₄ receptor seems to affect immune systems both positively and negatively. For example, EP₄ receptor-stimulated differentiation of T_H1 cells and production of IL-23 in dendritic cells and resulting inflammation in experimental autoimmune encephalomyelitis were reported (Yao *et al.* 2009). However, in microglia, the activation of EP₄ receptor was reported to suppress the LPS-stimulated production of pro-inflammatory cytokines (Shi *et al.* 2010).

As described in the introduction, NSAIDs have attracted considerable attention as a new class of drugs for the treatment and prevention of AD, although it should also be noted that some clinical studies have recorded negative results (Imbimbo *et al.* 2010). NSAIDs can be classified into two groups: newly developed COX-2-specific NSAIDs (such as celecoxib) and classical NSAIDs without COX-2 specificity (such as indomethacin). The clinical use of classical NSAIDs is associated with gastrointestinal side effects (Hawkey 2000), as a result of the strong protective effect of prostaglandins on the gastroin-

testinal mucosa (Vane and Botting 1996). Given that it is mainly COX-1, which is expressed in this mucosa, COX-2-specific NSAIDs cause less of an effect on prostaglandin levels in this region, and therefore produce fewer gastrointestinal side effects than classical NSAIDs. However, it has recently been shown that clinical use of COX-2-specific NSAIDs is associated with cardiovascular thrombotic side effects (Ray *et al.* 2004; Singh 2004). These side effects of NSAIDs are likely to prove problematic if the drugs are used long term for the prevention or treatment of AD.

Compared with NSAIDs, we consider that EP₄ receptor-specific antagonists have advantages in relation to both safety and efficacy, based on the following lines of evidence. EP₁ and EP₃ receptors have been reported to be involved in PGE₂-mediated protection of the gastrointestinal mucosa by stimulating the production of bicarbonate and gastric mucosal blood flow, respectively (Takeuchi *et al.* 1997; Araki *et al.* 2000). Therefore, antagonists specific for the EP₄ receptor would be gastrointestinally safer than NSAIDs. However, it is now believed that inflammation has both positive and negative effects in relation to the progression of AD; for example, inflammation activates the phagocytosis of A β by microglia (Shaftef *et al.* 2007; Chakrabarty *et al.* 2010). However, NSAIDs that inhibit overall inflammation inactivate microglial phagocytosis (Yan *et al.* 2003). Therefore, compared with general anti-inflammatory agents, inhibitors that specifically act on the inflammation-mediated progression of AD may be more effective. NSAIDs suppress inflammation through both COX-dependent and COX-independent mechanisms, such as activation of the peroxisome proliferators activated receptor- γ and inhibition of nuclear factor-kB (Tegeeder *et al.* 2001), with COX-mediated inhibition and the resulting decrease in PGE₂ levels seen to play a major role in the anti-AD activity of NSAIDs (Qin *et al.* 2003; Heneka *et al.* 2005). Furthermore, it was recently reported that the ability of NSAIDs to decrease PGE₂ levels is important in NSAID-dependent protection of hippocampal LTP against A β toxicity and restoration of A β -mediated suppression of synaptic plasticity and memory function (Kotilinek *et al.* 2008). Based on the findings of the present study, we consider that PGE₂ impairs cognitive performance at least partly through activation of the EP₄ receptor. Thus, we propose that EP₄ receptor-specific antagonists, such as AE3-208, will prove therapeutically more effective than NSAIDs as a result of their greater safety and efficacy. However, although we previously suggested that EP₁ and EP₃ receptors are not involved in PGE₂-stimulated production of A β *in vitro*, it is not clear whether activation of EP₁ and EP₃ receptors affect cognitive performance. Furthermore, modulation of COX-2 expression by activation of EP₄ receptor was also suggested (Shi *et al.* 2010). Therefore, the mechanism by which PGE₂ modulates cognitive performance is unclear at present and understanding of such mechanism is important for the identification of other targets of AD drugs.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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Purification and characterization of HSP-inducers from *Eupatorium lindleyanum*

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ABSTRACT

The expression of heat shock proteins (HSPs), particularly HSP70, provides resistance to stressors. We recently reported that ultraviolet (UV)-induced melanin production and skin damage were suppressed in transgenic mice expressing HSP70 and that an extract of *Eupatorium lindleyanum* induces the expression of HSP70 in cells. Here we report the purification of eupalinolide A and B (EA and EB) from *E. lindleyanum*, and describe their actions as HSP-inducers. EA and EB both induced the expression of HSP70 in cells at concentrations that did not significantly affect cell viability. Treatment of cells with EA or EB activated heat shock factor 1 (HSF1), while the artificial suppression of HSF1 expression diminished the EA- or EB-mediated induction of HSP70 expression. Furthermore, EB inhibited the interaction between HSF1 and HSP90, which is known to inhibit the activity of HSF1. These findings suggest that EA and EB induce the expression of HSP70 via the activation of HSF1 by inhibiting the interaction between HSF1 and HSP90. EA and EB both induced the expression of HSP70 synergistically with other stressors. Furthermore, pre-treatment of cells with EA or EB suppressed melanin production and stressor-induced apoptosis. These effects were suppressed by the artificial suppression of HSP70 expression. *In vivo*, the percutaneous administration of EB induced the expression of HSP70 and suppressed UVB radiation-induced damage, inflammatory responses and melanin production in the skin. These results suggest that EA and EB could be beneficial for use in cosmetics and medicines as a consequence of their inhibitory action on UV-induced skin damage and melanin production.

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1. Introduction

In addition to changes with aging, the skin is damaged by various environmental stressors, especially by solar ultraviolet (UV) radiation (photo-aging). UV light can be separated according to wavelength into UVA (320–400 nm), UVB (290–320 nm) and UVC (100–290 nm) [1]. As most UVC light from the sun is absorbed by the ozone layer, and the cell damaging effect of UVA is relatively weak, it seems therefore that UVB plays a central role in photo-aging [2–5].

Abbreviations: ANOVA, analysis of variance; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGA, geranylgeranylacetone; HSF1, heat shock factor 1; HSP, heat shock protein; IBMX, 3-isobutyl-1-methylxanthine; MITF, microphthalmia-associated transcription factor; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF- κ B, nuclear factor kappa B; UV, ultraviolet.

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UV-induced skin damage (such as erythema, plaque-like thickening, loss of skin tone, deep furrowing and fine wrinkle formation) is caused not only by direct damage to the skin but also indirectly via the induction of inflammation [6]. UV radiation also induces the development of skin cancer (photo-carcinogenesis) through DNA damage [5]. Furthermore, UV-induced skin hyperpigmentation disorders due to abnormal melanin production cause clinical and cosmetic problems. UV-induced melanin production is mediated by a cAMP-dependent pathway in which the exposure of keratinocytes to UV light stimulates the release of signal molecules, which in turn elevate the intracellular cAMP level and induce the expression of tyrosinase [7]. Tyrosinase is a rate-limiting enzyme in melanin synthesis and its expression is positively regulated by microphthalmia-associated transcription factor (MITF) [7]. Therefore, chemicals and natural products that suppress the activity and/or expression of tyrosinase could potentially be pharmaceutically and cosmetically beneficial as hypopigmenting agents. However, the UV-induced modest production of melanin plays an important role in protecting the skin against UV-dependent damage, because melanin acts as a filter to limit the penetration of UV into the epidermis and dermis [8]. Thus,

to develop hypopigmenting agents (skin whitening agents) without worsening UV-induced skin damage, it is important not only to suppress melanin production but also to protect the skin from UV-induced damage.

When cells are exposed to stressors, a number of so-called stress proteins are induced that confer protection against such stressors. HSPs are representative of these stress proteins and their cellular up-regulation of expression, especially that of HSP70, provides resistance as a consequence of the HSPs' capacity to re-fold or degrade denatured proteins produced by stressors [9]. The induction of HSP expression by stressors is achieved at the transcription level via heat shock factor 1 (HSF1), a transcription factor [10]. Furthermore, the stressor-induced multistep activation of HSF1, such as phosphorylation, oligomerization to a trimeric state and re-localization into the nucleus, has been identified [11]. In addition to its cytoprotective effects, HSP70 exerts an anti-inflammatory action via its inhibitory effect on nuclear factor kappa B (NF- κ B) [12–15]. Since stressor-induced tissue damage and inflammation are involved in various diseases, HSPs and HSP-inducers have received much attention for their therapeutic potential [11]. Geranylgeranylacetone (GGA) is a leading anti-ulcer drug on the Japanese market; it has been described as a non-toxic HSP-inducer and was recently shown to suppress inflammatory bowel disease-related experimental colitis, as well as lesions of the small intestine and pulmonary fibrosis in mice [16–20].

It is known that various HSPs are constitutively expressed in the skin and that their expression, especially that of HSP70, is up-regulated by stressors such as heat-shock and UV radiation [21,22]. While the artificial expression of HSP70 in keratinocytes and melanocytes confers protection against UV *in vitro* [21,23–26], the role of HSP70 in photo-aging *in vivo* has remained unclear. We recently showed that UV radiation-induced skin damage (such as epidermal apoptosis) and the resulting inflammatory response were suppressed in transgenic mice expressing HSP70. We also found a lower level of UV-induced DNA damage in the transgenic mice than in control wild-type mice [27]. These results suggest that the protective action of HSP70 against UV radiation-induced skin damage is mediated by processes that attenuate apoptosis, inflammation and DNA damage. We also recently reported that melanin production in cultured mouse melanoma (B16) cells was suppressed by the overexpression of HSP70 and that this suppression is mediated by the modulation of MITF activity through a direct interaction between HSP70 and MITF [28]. *In vivo*, a UV radiation-induced increase in the amount of melanin in the skin was suppressed in transgenic mice expressing HSP70 [28]. Taken together, these results suggest that HSP70 inducers could be beneficial for use in hypopigmenting cosmetics and medicines, functioning by suppressing melanin production while simultaneously protecting the skin against UV-induced damage.

We searched for HSP70 inducers from Chinese herbs and found that an ethanol extract of *Eupatorium lindleyanum* (*E. lindleyanum*) can induce expression of HSP70 in B16 cells [29]. *E. lindleyanum* has been used in China and other Asian countries as a traditional medicine for the treatment of cough, fever and tracheitis based on its anti-microbial and anti-inflammatory activities [30–32]. Furthermore, it was reported that *E. lindleyanum* contains ingredients such as volatile oils, flavonoids, alkaloids, coumarins, sesquiterpenes and esters [33], although the specific molecules responsible for the pharmacological activities of *E. lindleyanum* have not been identified. In this study, we describe our efforts to purify molecules responsible for *E. lindleyanum*'s HSP70-inducing properties. We identified two compounds, eupalinolide A and B (EA and EB), both of which are capable of inducing HSP70 expression in cells at concentrations that do not affect cell viability. Our results suggest that both EA and EB induce the expression of HSP70 via the activation of HSF1, and that both these compounds suppress

stressor-induced apoptosis and melanin production by inducing HSP70 expression. Furthermore, *in vivo* results showed that the percutaneous administration of EB induces the expression of HSP70 and suppresses UVB-induced cell damage, inflammatory responses and melanin production in the skin, suggesting that these molecules could be beneficial for use in cosmetics and medicines.

2. Materials and methods

2.1. Materials and animals

The MCI-gel CHP20P column was from Mitsubishi Chemical (Tokyo, Japan) and Chromatorex ODS column was from Fuji Silysia Ltd. (Aichi, Japan). Optimal cutting temperature (OCT) compound was from Sakura Fintech (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Wako Pure Chemical Industries (Osaka, Japan). The RNeasy Fibrous Tissue Mini kit and HiPerFect transfection reagent were obtained from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was from Takara (Shiga, Japan), and the iQ SYBR Green Supermix was from Bio-Rad Laboratories (Hercules, CA). Fetal bovine serum (FBS), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), menadione and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich Co. (St. Louis, MO). Dynabeads Protein G, surface-activated Dynabeads, Lipofectamine (TM2000) and Alexa Fluor 594 goat anti-mouse immunoglobulin G were purchased from Life Technologies Co. (Carlsbad, CA). Antibodies against tyrosinase, I κ B- α , lamin B and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and antibody against HSP70 was from R&D Systems (Minneapolis, MN). Antibodies against HSP25, HSP47, HSP60 and HSP90 were from Enzo Life Sciences Inc. (Farmingdale, NY). Antibody against MITF was obtained from Thermo Scientific (Waltham, MA). Antiserum against HSF1 and HSP40 and the HSF1 expression vector pcDNA3.1/hHSF1 were kindly provided by Dr. Akira Nakai (Yamaguchi University) [34]. L-DOPA was from Nacalai Tesque (Kyoto, Japan). Mayer's hematoxylin, 1% eosin alcohol solution and malinol were from Muto Pure Chemicals (Tokyo, Japan). VECTASHIELD was from Vector Laboratories (Burlingame, CA). 4',6-Diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The whole plant of *E. lindleyanum* was from Maruzen Pharmaceuticals (Hiroshima, Japan). Transgenic mice expressing HSP70 and their wild-type counterparts (6–8 weeks old, male) were a gift from Drs. C.E. Angelidis and G.N. Pagoulatos (University of Ioannina, Ioannina, Greece) and were prepared as described previously [35]. Homozygous transgenic mice were used in these experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care Committee of Keio University and Kumamoto University.

2.2. Extraction, fractionation and identification of EA and EB

The whole plant of *E. lindleyanum* (10.1 g) was extracted three times with 100% ethanol by refluxing for 2 h and the extract was concentrated *in vacuo* to afford residues (499 mg). This extract was partitioned with 90% methanol and *n*-hexane to give two fractions (90% methanol: 318 mg, *n*-hexane: 181 mg). Most of the HSP70-inducing activity (as determined by immunoblotting analysis) was recovered in the 90% methanol fraction, which was loaded onto a polystyrene gel column (MCI-gel CHP20P column (\emptyset 17 mm \times 100 mm)) and eluted by the stepwise addition of H₂O, 50% methanol, 100% methanol and 100% acetone. Most of the HSP70-inducing activity was recovered in the 100% methanol

fraction (120 mg), which was further separated using a Chromatorex ODS column (Ø 15 mm × 150 mm) with a stepwise elution of 50% methanol, 75% methanol and 100% methanol. The active fraction (50% methanol fraction) (120 mg) was separated by reverse-phase high performance liquid chromatography (HPLC) (Cosmosil AR-II ODS, Ø 20 mm × 250 mm) to yield EA (fraction 3, 4.6 mg, MW: 463.2) and EB (fraction 7, 4.5 mg, MW: 463.2). The structures of molecules in these fractions were identified on the basis of their NMR spectra and MS data as previously reported [33].

2.3. Cell culture

We used the PAM212 mouse squamous cell carcinoma and B16 mouse melanoma cell lines in this study. PAM212 and B16 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C. Transfection with pcDNA3.1/hHSF1 was carried out using Lipofectamine (TM2000) according to the manufacturer's protocol. Cell viability was determined by the MTT method [36]. Fluorescence-activated cell sorting analysis (FACS) for the measurement of apoptotic cells was performed as described previously [36].

2.4. siRNA targeting of genes

The siRNAs for HSF1 and HSP70 and nonspecific siRNA were purchased from Qiagen. Cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer's instructions.

2.5. Immunoblotting analysis

Whole cell and nuclear extracts were prepared as described previously [36]. The protein concentration of each sample was determined by the Bradford method [37]. Samples were applied to polyacrylamide SDS gels and subjected to electrophoresis, after which proteins were immunoblotted with each antibody.

2.6. Determination of melanin content in vitro

The melanin content of cells was determined as described previously [38,39] with some modifications. Cells were homogenized with 1 N NaOH and the melanin content was determined by measuring the absorbance at 405 nm with a plate reader (Fluostar Galaxy; BMG Labtech, Germany).

2.7. Tyrosinase activity assay

Tyrosinase activity was assayed as described previously [40] with some modifications. Cells were washed with phosphate-buffered saline (PBS) and homogenized with 20 mM Tris/HCl (pH 7.5) buffer containing 0.1% Triton X-100. Tyrosinase activity (as indicated by oxidation of L-DOPA to DOPACHrome) was monitored as follows: cell extracts (50 µl) were mixed with 100 µl of freshly prepared substrate solution (0.1% L-DOPA in PBS) and incubated at 37 °C. The production of DOPACHrome was monitored by measuring the absorbance at 475 nm with a plate reader (Fluostar Galaxy) and corrected for the auto-oxidation of L-DOPA.

2.8. Real-time RT-PCR analysis

Real-time RT-PCR was performed as previously described [41] with some modifications. Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 µg RNA) were reverse-transcribed using a first-strand cDNA

synthesis kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad Laboratories)) experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software (Bio-Rad Laboratories). Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers were designed using the Primer3 website. Mouse primers were (name: forward primer, reverse primer): *hsp25*: 5'-cctctccctatcccctgag-3', 5'-ttggctccagactgttcaga-3'; *hsp40*: 5'-ctccagtcacccatgacctt-3', 5'-tgctcttccatcagggttc-3'; *hsp47*: 5'-caaccctttgaccaagaca-3', 5'-tgattatctcgcaccaggaa-3'; *hsp60*: 5'-cgttgc-caataacacaaacg-3', 5'-cttcaggggtgtcacaggt-3'; *hsp70.1*: 5'-tgcacttgatagctgcttgg-3', 5'-cagtcacgcaattacctaagaa-3'; *hsp70.3*: 5'-ggccttgaggactgtcattatt-3', 5'-cccactgcaataacaaaag-3'; *hsp90α*: 5'-aaaggcagaggctgacaaga-3', 5'-agggaggcattctctcagt-3'; *hsp90β*: 5'-gcggca-aagacaagaaaaag-3', 5'-gaagtggtctctccagtcac-3'; *hsp70*: 5'-tgggtgtg-acgaagatgaag-3', 5'-aggtcgaagatgagcaggtt-3'; *tyrosinase*: 5'-cctcctg-gcagatcattgt-3', 5'-ggtttggcttgcattgt-3'; *tyrp1*: 5'-tggaccaatcag-gagaaacc-3', 5'-atacacggacctccaagcac-3'; *dct*: 5'-tgtgcaagattgcctgt-ctc-3', 5'-agtccagtggtccgctctct-3'; *mitf*: 5'-ctagagcgcattggacttcc-3', 5'-acaagttcctggctgcagtt-3'; *gapdh*: 5'-aacttggcattgtggaag-3', 5'-acacattgggggtggaaca-3'.

2.9. Co-immunoprecipitation assay

Immunoprecipitation was carried out as described previously [42] with some modifications. Cells were harvested, lysed with lysis buffer (10 mM Tris/HCl (pH 7.5) buffer containing 0.1% NP-40 and 150 NaCl) and centrifuged. The antiserum against HSF1 was added to the supernatants and the samples were incubated for 2 h at 4 °C with rotation. Dynabeads Protein G was added and the samples were incubated for 1 h at 4 °C with rotation. Beads were washed with lysis buffer four times and proteins were eluted by boiling in SDS sample buffer.

2.10. Preparation of EB-fixed beads and pull-down assay

Immobilization of EB to surface-activated Dynabeads was carried out according to the manufacturer's protocol. Cells were harvested, lysed and centrifuged. EB-fixed beads were added to the supernatants and the samples were incubated for 2 h at 4 °C with rotation. Beads were washed four times with the lysis buffer (see above) and proteins were eluted by boiling in SDS sample buffer.

2.11. UVB irradiation

Animals and cultured cells were exposed to UVB radiation with a double bank of UVB lamps (peak emission at 312 nm, VL-215LM lamp, Vilber Lourmat, Paris, France). The UV energy was monitored by a radiometer sensor (UVX-31, UV Products, Cambridge, UK). Animals were placed under deep anesthesia with chloral hydrate (250 mg/kg) before each irradiation. Fur was removed with electric clippers prior to the first irradiation.

2.12. MPO activity

Myeloperoxidase (MPO) activity in the skin was measured as described previously [35]. Animals were placed under deep ether anesthesia and killed. The skin was dissected, rinsed with cold saline and cut into small pieces. Samples were homogenized in 50 mM phosphate buffer and centrifuged. MPO activity was determined in 10 mM phosphate buffer with 0.5 mM *o*-dianisidine, 0.00005% (w/v) hydrogen peroxide and 20 µg of protein. MPO activity was obtained from the slope of the reaction curve, and its

specific activity was expressed as the number of hydrogen peroxide molecules converted per minute and per mg of protein.

2.13. Histological and immunohistochemical analyses

For histological examination (hematoxylin and eosin staining), skin samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4- μ m-thick sections, which were then deparaffinized and washed in PBS. Sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected using a BX51 microscope (Olympus; Tokyo, Japan). Fontana-Masson staining was performed as previously described [43]. The intensity of Fontana-Masson staining in the epidermis was measured by LuminaVision (Mitani Corporation, Tokyo, Japan).

For HSP70 immunohistochemical analysis, skin tissue samples were embedded in OCT compound and cryosectioned (4- μ m-thick sections). Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against HSP70 (1:200 dilution) and finally incubated for 2 h with Alexa Fluor 594 goat anti-mouse immunoglobulin G in the presence of DAPI (5 μ g/ml). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

2.14. Assay for melanin production in vivo

Skin reflective colorimetric measurements were assessed with a narrow-band simple reflectance meter (Mexameter MX18, Courage-Khazaka, Germany). The measurement was performed for three areas of skin, and the mean value was calculated. The measurement area was 5 mm in diameter, and the instrument was calibrated using black and white calibration plates.

2.15. Statistical analysis

All values are expressed as the mean \pm standard deviation (S.D.) or standard error of the mean (S.E.M.). Two-way analysis of variance (ANOVA) followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Purification of HSP-inducers from *E. lindleyanum* extract

As mentioned in the introduction, we recently reported that an ethanol extract of *E. lindleyanum* shows HSP-inducing activity, particularly with regard to HSP70 [29]. By partition with 90% methanol and *n*-hexane, polystyrene gel column chromatography and ODS column chromatography, we carried out the purification of molecules responsible for the HSP70-inducing activity, which was monitored by immunoblotting experiments (Fig. 1A). In the final step of the purification process, we obtained 12 fractions from HPLC experiments and examined their HSP70-inducing and cytotoxic activities (Fig. 1B). HSP70-inducing activity was recovered in fractions 3 and 7, whereas cytotoxic activity was recovered in fractions 2 and 6 (Fig. 1B). ^3H and ^{13}C NMR analyses were performed and the presence of EA in fraction 3 and EB in fraction 7 was identified on the basis of previously reported NMR spectra and MS data [33] (Fig. 1C). While both EA and EB were identified as major sesquiterpenes in *E. lindleyanum* [33], their biological activities have not been examined.

Considering the interesting applications that HSP-inducers could have in medicines and cosmetics, it is important that they are able to induce the expression of HSPs without decreasing cell

viability. As shown in Fig. 1D, the induction of HSP70 expression in B16 cells was apparent when EA or EB was employed at a concentration of 5 μ g/ml, with cell viability remaining unaffected even at concentrations as high as 10 μ g/ml. On the other hand, while the ethanol extract of *E. lindleyanum* induced the expression of HSP70, there was a concomitant slight decrease in cell viability (Fig. 1D), suggesting that the cytotoxic effect of the ethanol extract is not due to EA or EB. Time-course experiments showed that the induction of HSP70 expression by EA or EB was apparent within 6 h of the beginning of incubation of B16 cells with these compounds (Fig. 1E).

The induction of HSP70 expression by both EA and EB was also observed in the mouse keratinocyte cell line (PAM212 cells), again at concentrations that did not affect cell viability (Fig. 2A).

We also examined the effect of EA and EB on the expression of HSPs other than HSP70 in PAM212 cells. Both EA and EB induced HSP25, HSP40 and HSP90 expression but not that of HSP47 or HSP60 (Fig. 2B). Similar results were observed at the mRNA level (Fig. 2C). The antibody against HSP40, HSP70 or HSP90 that was used in the experiments reported in Fig. 2B can recognize Hdj1 isoform of HSP40, both HSP70.1 and HSP70.3, or both HSP90 α and HSP90 β , respectively. The results presented in Fig. 2C show that expression of mRNAs corresponding to all these proteins except HSP90 β was induced by EA or EB. Since it was reported that the expression of HSP25 in keratinocytes plays an important role in their differentiation [44], the results in Fig. 2B and C suggest that EA or EB could affect this process.

3.2. Mechanism for the induction of HSP70 expression by EA and EB

To understand the mechanism governing the induction of HSP70 expression by EA or EB, we first examined the contribution of HSF1 by using RNA interference. As shown in Fig. 3A, transfection of cells with siRNA for HSF1 suppressed not only the expression of HSF1 but also the EA- and EB-induced expression of HSP70, showing that HSF1 is important for this induction. We also examined the phosphorylation of HSF1 and re-localization of HSF1 into the nucleus in the presence of EA or EB. The phosphorylation of HSF1 can be detected as an upward band-shift [45,46], and treatment of cells with heat-shock caused such an upward band-shift (Fig. 3B) and an increase in HSF1 in the nuclear fraction (Fig. 3C), thus demonstrating the phosphorylation and re-localization of HSF1 into the nucleus. Although the extent to which this took place was not as clear-cut as that seen with heat-shock, phosphorylation and re-localization of HSF1 into the nucleus was also observed in cells treated with EA or EB (Fig. 3B and C). Furthermore, EA- or EB-mediated phosphorylation seems to be transient, because phosphorylation was not observed in cells treated with EA or EB for 24 h (Fig. 3A). Taken together, the results presented in Fig. 3 suggest that EA and EB induce the expression of HSP70 via the activation of HSF1.

HSF1 is normally maintained in a latent form by virtue of its association with HSP90 [47]. We therefore tested whether EA or EB activates HSF1 by dissociation of HSF1 from HSP90. Whole cell extracts were prepared from PAM212 cells treated with EA, EB or heat-shock, and the binding of HSP90 to HSF1 was monitored by a co-immunoprecipitation assay in which we immunoprecipitated HSF1 and looked for the presence of HSP90. In these experiments, we used PAM212 cells overproducing HSF1. The efficient precipitation of HSF1 was observed in a manner that was dependent on the overexpression of HSF1 (Fig. 4A). In control extract, HSP90 was co-immunoprecipitated; however, this co-immunoprecipitation was not observed in extracts prepared from cells treated with EA, EB or heat-shock (Fig. 4A), suggesting that these treatments inhibit the binding of HSP90 to HSF1.

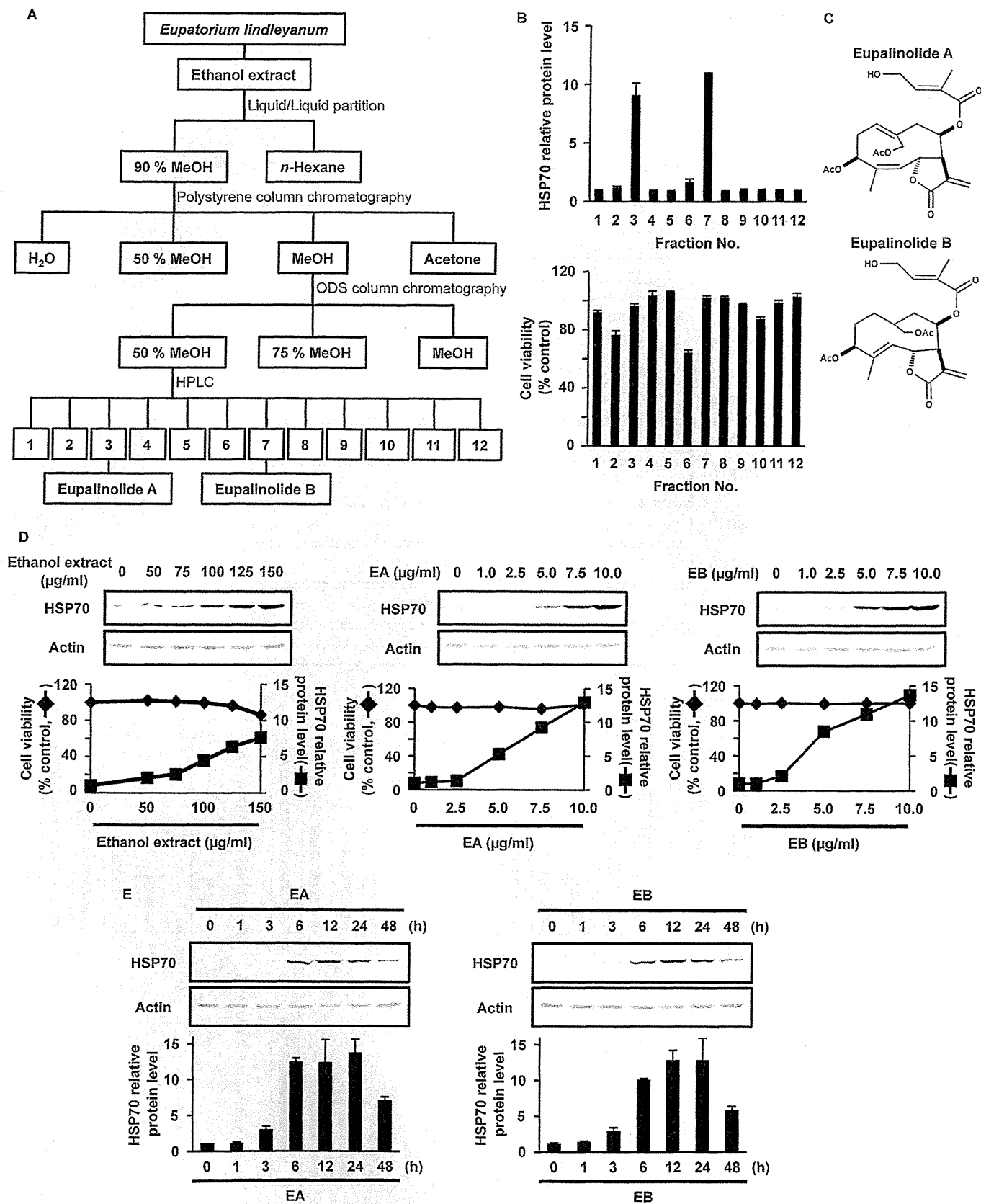


Fig. 1. Purification and characterization of EA and EB. Procedure for purification of EA and EB from *E. lindleyanum* is outlined in (A) (details are described in Section 2). B16 cells were incubated for 24 h with 7.5 µg/ml of each HPLC fraction and expression of HSP70 and cell viability were determined by immunoblotting and the MTT method, respectively (B). Structures of EA and EB are shown in (C). B16 cells were incubated with indicated concentrations of an ethanol extract of *E. lindleyanum*, EA or EB (D) or 10 µg/ml EA or EB (E) for 24 h (D) or indicated periods (E). Cell viability was determined by the MTT method and whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin (D and E). The intensity of the HSP70 band relative to the actin band is shown (D and E). Values are given as mean ± S.D (n = 3).

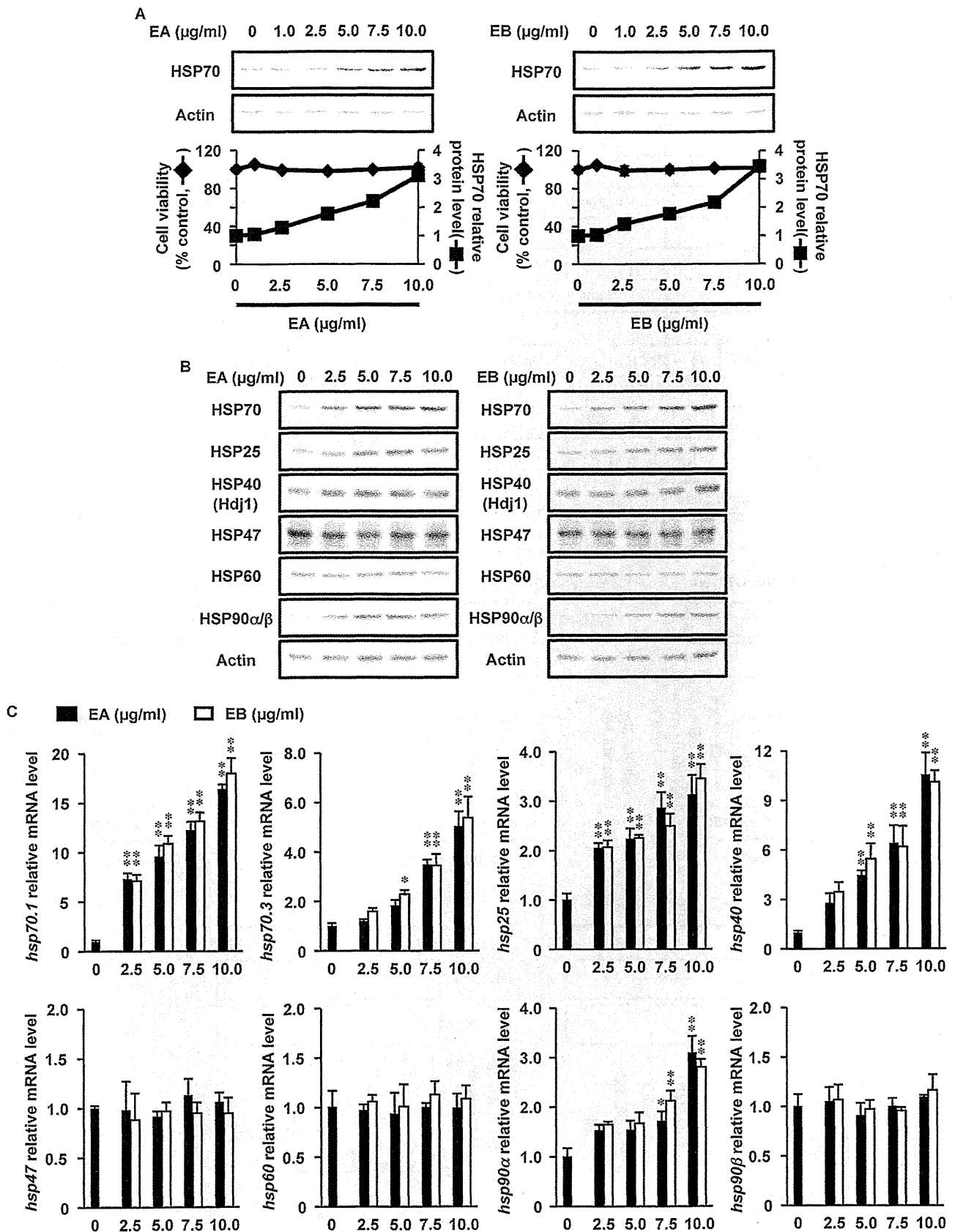


Fig. 2. Effects of EA and EB on HSP70 expression in PAM212 cells. PAM212 cells were incubated with indicated concentrations of EA or EB for 24 h (A and B) or 3 h (C). HSP70 expression and cell viability were determined as described in the legend of Fig. 1 (A). Whole cell extracts were analyzed by immunoblotting with an antibody against each protein (B). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values were normalized to *gapdh* gene expression and expressed relative to the control sample (C). Values are given as mean ± S.D. (n = 3). **P < 0.01; *P < 0.05.

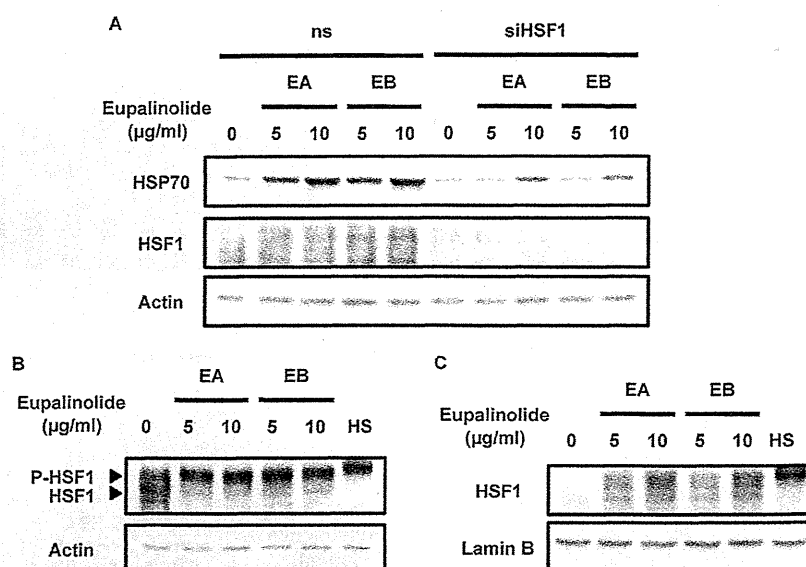


Fig. 3. HSF1 activation by EA and EB. PAM212 cells were transfected with siRNA for HSF1 (siHSF1) or nonspecific siRNA (ns) and incubated for 48 h. Cells were further incubated with indicated concentrations of EA or EB for 24 h (A). PAM212 cells were incubated with indicated concentrations of EA or EB for 3 h (B) or 1 h (C) or heat-shocked for 1 h at 43 °C (HS) (B and C). Whole cell extracts (A and B) or nuclear extracts (C) were analyzed by immunoblotting with an antibody against each protein (P-HSF1, hyperphosphorylated form of HSF1).

Finally, we tested the notion that EA or EB directly bind to HSP90 or HSF1 to inhibit their physical interaction. We prepared EB-fixed beads and after incubation of the beads with whole cell extract prepared from PAM212 cells cultured in normal conditions, collected the beads and looked for the presence of HSP90 or HSF1. As shown in Fig. 4B, HSP90 was detected in the fraction of EB-fixed beads and less clearly in that of control beads. We could not detect HSF1 in either fraction (data not shown). The detection of HSP90 in the EB-fixed bead fraction was suppressed when the incubation

was carried out in the presence of excess amounts of free EB (Fig. 4B). Furthermore, HSP90 could be extracted from EB-fixed beads with buffer containing excess amounts of free EB but not with control buffer. The extent of extraction was similar to that achieved by boiling the beads with SDS-sample buffer (Fig. 4C). These results suggest that EB specifically binds to HSP90. We could not prepare EA-fixed beads due to the limited amount of EA available.

We also examined the synergistic effect of EA and EB with ethanol, a representative stressor that induces the expression of HSPs, on the induction of HSP70 expression. To observe the synergistic effect, a concentration of EA, EB or ethanol should be used that does not clearly induce the expression of HSP70 by itself. On this basis we used a concentration of 1–2 µg/ml for EA and EB (based on the data in Fig. 2A) or 3.5% ethanol (based on the dose-response profile of the induction of expression of HSP70 by ethanol in PAM212 cells (data not shown)). As shown in Fig. 5A and B, treatment of cells with 1–2 µg/ml EA (or EB) or 3.5% ethanol alone did not significantly up-regulate the expression of HSP70; however, a combination of both treatments clearly did induce HSP70 expression (Fig. 5A and B). Similar results were observed at the mRNA level (Fig. 5C). These results suggest that EA and EB act synergistically with other stressors such as ethanol to induce the expression of HSP70.

To examine the mechanism of this synergistic action, we examined the effect of 1–2 µg/ml EA or EB on the phosphorylation and re-localization into the nucleus of HSF1. As shown in Fig. 5D, treatment of cells with 1–2 µg/ml EA (or EB) but not 3.5% ethanol increased the phosphorylated form of HSF1, while their combined use resulted in a much higher level of phosphorylation. On the other hand, when cells were treated with 1–2 µg/ml EA (or EB) or 3.5% ethanol alone, re-localization of HSF1 into the nucleus was not stimulated; however, when they were used in combination re-localization of HSF1 into the nucleus did occur (Fig. 5E). These results suggest that the synergistic effect of EA (or EB) used in combination with other stressors to induce the expression of HSP70 is mediated by the stimulation of HSF1 phosphorylation. We also found that the simultaneous treatment of cells with EA or EB suppressed ethanol-induced cell death (Fig. 5F).

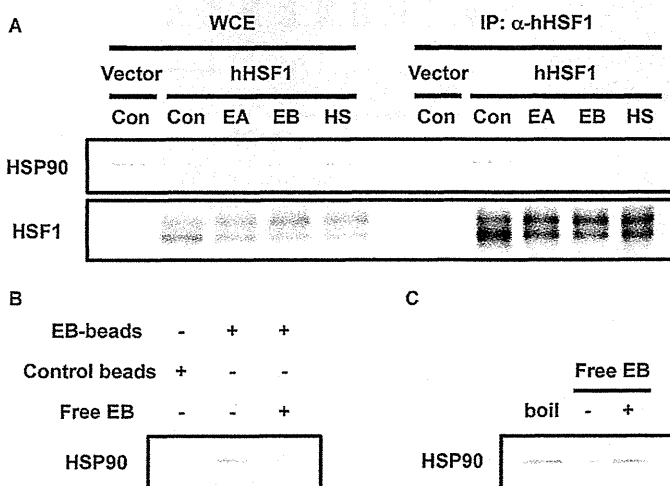


Fig. 4. Dissociation of HSF1 from HSP90 by EA and EB. PAM212 cells were transiently transfected with the expression plasmid for human HSF1 (pcDNA3.1/hHSF1) or vector (pcDNA3.1). After 24 h, cells were incubated with 5 µg/ml of EA or EB for 3 h or heat-shocked for 1 h at 43 °C (HS). Whole cell extracts (WCE) were immunoprecipitated with an antibody against HSF1 (IP: α -hHSF1) and both fractions were analyzed by immunoblotting with an antibody against each protein (A). Whole cell extracts prepared from PAM212 cells (normal culture conditions) were incubated with EB-conjugated magnetic beads (EB-beads) or control beads for 2 h in the presence or absence of free EB (0.5 mg/ml) and beads were collected. Samples were analyzed by immunoblotting with an antibody against HSP90 (B). The samples purified from whole cell extracts with EB-conjugated magnetic beads (described above) were suspended in the lysis buffer with or without free EB (0.5 mg/ml) or boiled in SDS-sample buffer. Supernatants were analyzed by immunoblotting with an antibody against HSP90 (C).

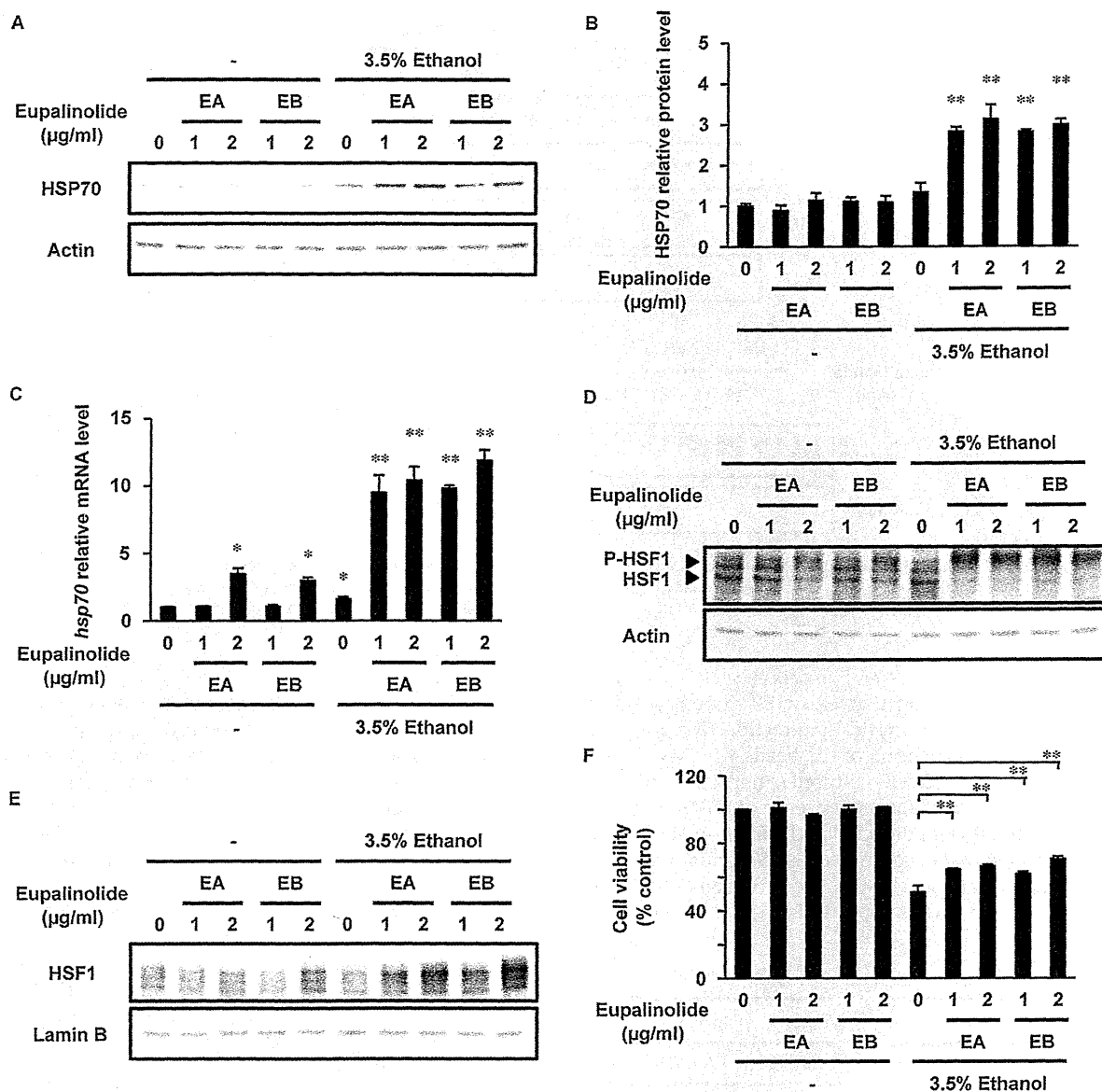


Fig. 5. Synergistic effects of EA and EB with ethanol on induction of HSP70 expression. PAM212 cells were incubated with indicated concentrations of EA or EB in the presence or absence of 3.5% ethanol for 24 h (A, B and F), 3 h (C and D) or 1 h (E). Whole cell extracts (A and D) or nuclear extracts (E) were analyzed by immunoblotting as described in the legends of Figs. 1 and 3. The intensity of the HSP70 band relative to the actin band is shown (B). The *hsp70* mRNA expression was monitored as described in the legend of Fig. 2. Cell viability was determined by the MTT method (F). Values are given as mean \pm S.D. ($n = 3$). ** $P < 0.01$; * $P < 0.05$.

3.3. Pharmacological effects of EA and EB in vitro

As mentioned above, HSP70 exerts various actions in cells, such as cytoprotection and the suppression of melanin production. Thus, as the results described above suggest that EA and EB could have pharmacological activities through the induction of HSP70 expression, we tested the cytoprotective effects and melanin inhibition properties of EA and EB. In the results of experiments shown in Fig. 6, PAM212 cells were pre-treated with EA or EB for 6 h and then exposed to various stressors. We confirmed the induction of HSP70 expression after the 6 h incubation with EA or EB in PAM212 cells (Fig. 6A). This pre-treatment with EA or EB also increased cell viability after subsequent exposure to UVB radiation (Fig. 6B). FACS analysis showed that UVB radiation induced an increase in the number of apoptotic cells (cells in sub-G1), and that this was suppressed in cells pre-treated with EA or EB (Fig. 6C). To test the contribution of HSP70 to this EA- or EB-mediated cytoprotection against UVB, we used siRNA for HSP70. Transfection of this siRNA suppressed the cytoprotective effect of EA or EB

against UVB radiation but did not affect cell viability in the absence of UVB (Fig. 6D). These results suggest that EA and EB protect cells from UVB radiation via the induction of HSP70 expression.

We performed similar experiments for other stressors. As shown in Fig. 6E and G, pre-treatment of cells with EA or EB increased cell viability after subsequent exposure to menadione (a superoxide anion-releasing drug) or heat-shock. Furthermore, FACS analysis showed that apoptosis induced by each of these stressors was suppressed in cells pre-treated with EA or EB (Fig. 6F and H).

We then examined the effect of EA and EB on UVB-induced melanin production in B16 cells. We used IBMX (a cAMP-elevating agent that acts by inhibiting phosphodiesterase) to mimic UVB-stimulated melanin production. As shown in Fig. 7A, treatment of cells with IBMX increased the melanin content in cells, while pre-treatment of cells with EA or EB suppressed this increase in a statistically significant manner. This finding indicates that EA and EB inhibit the IBMX-stimulated production of melanin. Transfection of cells with siRNA for HSP70 suppressed the inhibitory effect

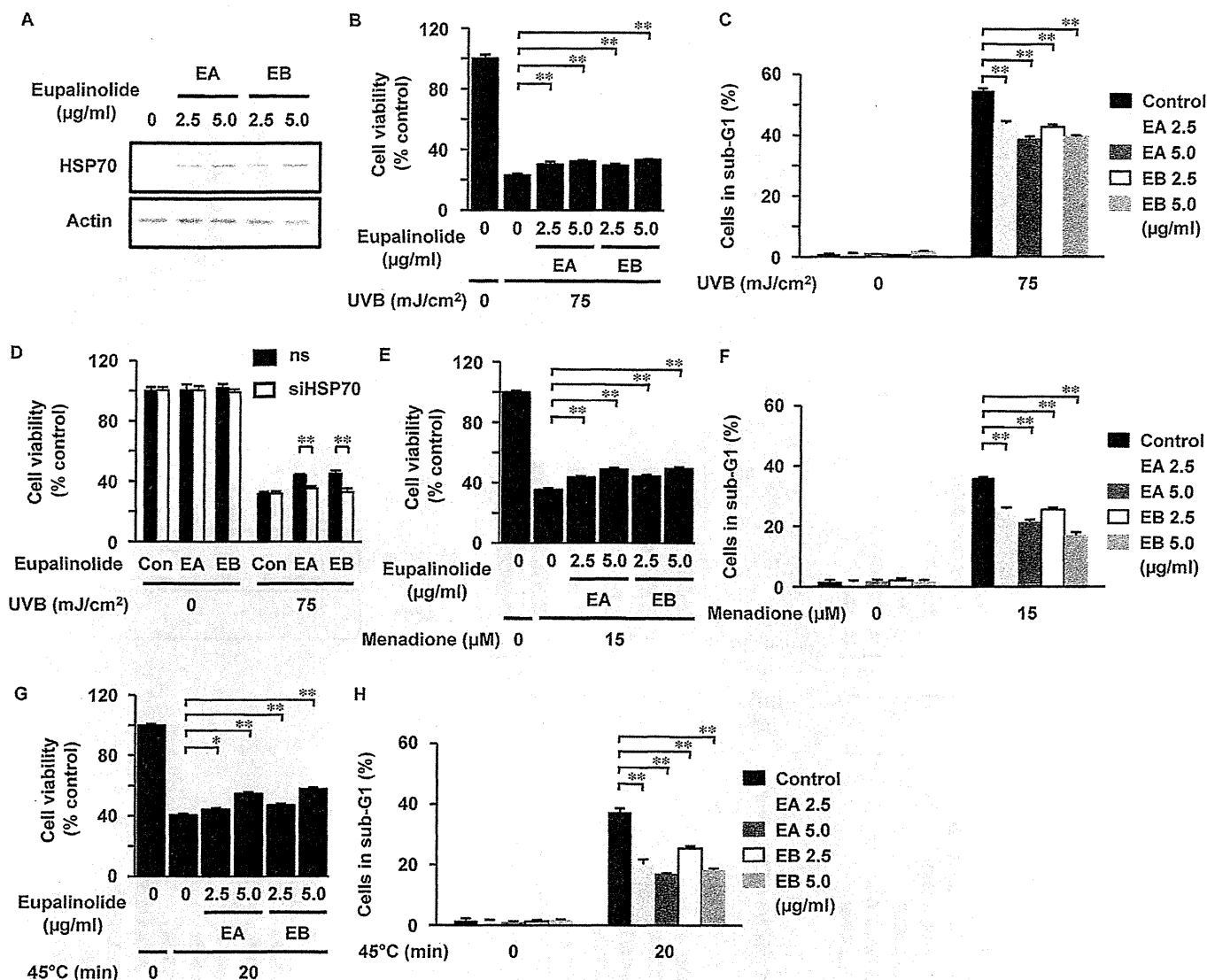


Fig. 6. Cytoprotective effects of EA and EB *in vitro*. PAM212 cells were pre-treated with indicated concentrations of EA or EB for 6 h, washed with fresh medium and cultured for 18 h. Cells were transfected with siRNA for HSP70 (siHSP70) or nonspecific siRNA (ns) and incubated for 48 h before the treatment with EA or EB described above (D). Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin (A). After treatment with EA or EB as described above, cells were irradiated with indicated doses of UVB and incubated for 24 h (B–D), incubated for 24 h with 15 µM menadione (E and F), or heat-shocked for 20 min at 45 °C followed by a recovery period at 37 °C for 24 h (G and H). Cell viability was determined by the MTT method (B, D, E and G). Apoptotic cells (cells in sub-G1) were counted by FACS as described in Section 2 (C, F and H). Values are given as mean ± S.D. ($n = 3$). ** $P < 0.01$; * $P < 0.05$.

of EA and EB on melanin production (Fig. 7B), supporting the contribution of HSP70 to this EA- or EB-mediated inhibition of melanin production.

To determine the mechanism governing the inhibition of melanin production by EA or EB, we turned our attention to examining tyrosinase activity. As shown in Fig. 7C, treatment of cells with IBMX increased the tyrosinase activity of cell extracts, while pre-treatment of cells with EA or EB decreased this activity in the presence of IBMX. On the other hand, when EA or EB was added directly to cell extracts prepared from IBMX-treated B16 cells, the tyrosinase activity was not significantly affected (Fig. 7D). This finding indicates that EA and EB affect the expression of tyrosinase. To test this further, we treated cells with IBMX and observed an increase in the level of tyrosinase protein, which was suppressed in cells pre-treated with EA or EB (Fig. 7E). Similar results were observed at the mRNA level (Fig. 7F), suggesting that EA and EB inhibit transcription of the *tyrosinase* gene.

As described in the introduction, MITF plays a central role in the UVB-induced expression of various genes, including that for

tyrosinase [48]. We examined the expression of two other MITF-regulated genes, tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (Dct). Expression of these genes was enhanced by IBMX in a manner that could be suppressed in cells pre-treated with either EA or EB (Fig. 7F). This finding suggests that EA and EB inhibit the MITF activity of cells.

We reported recently that overexpression of HSP70 in cells inhibited the transcriptional activity of MITF but not the expression of MITF [28]. We found here that treatment of cells with EA or EB affected neither protein nor mRNA levels of MITF (Fig. 7G and H). The results in Fig. 7 suggest that EA and EB inhibit the production of melanin by inducing the expression of HSP70.

3.4. Pharmacological effects of EA and EB *in vivo*

Finally, we tested the *in vivo* relevance of our *in vitro* results. To begin with, we examined the effect of EB on the expression of HSP70 in the skin by using immunohistochemical analysis with an antibody against HSP70 (we could not examine the effect of EA *in*

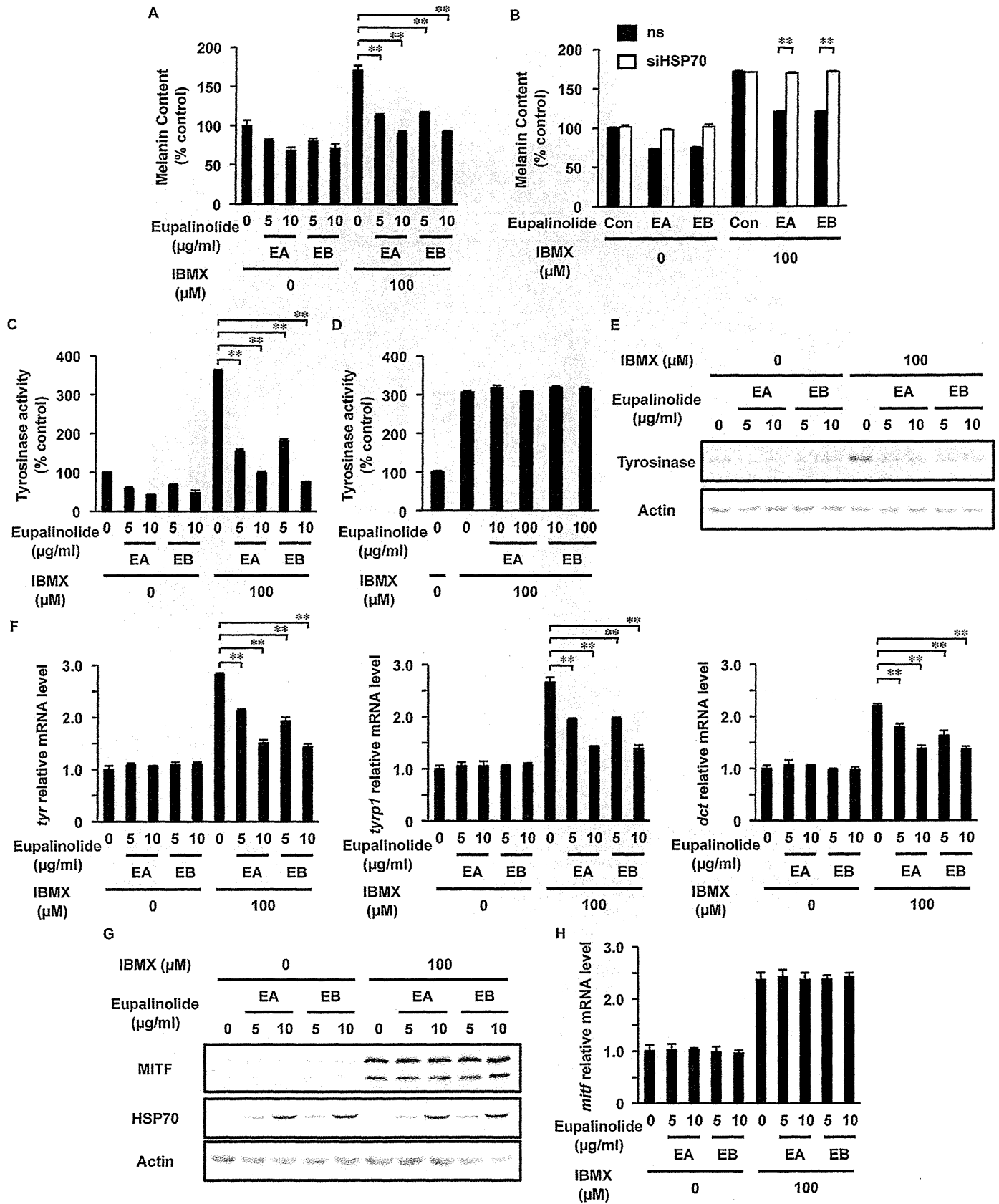


Fig. 7. Effects of EA and EB on melanin production *in vitro*. B16 cells were pre-incubated with indicated concentrations of EA, EB or ethanol extract of *E. lindleyanum* for 24 h (A–C, E–H). Cells were transfected with siRNA for HSP70 (siHSP70) or nonspecific siRNA (ns) and incubated for 48 h before the pre-treatment with EA or EB described above (B). After the pre-treatment procedure, cells were further incubated with or without 100 μM IBMX for 72 h (A, B), 48 h (C, E and F), 3 h (G) or 1.5 h (H). B16 cells were incubated with 100 μM IBMX for 48 h, and prepared whole cell extracts were mixed with EA or EB to obtain the indicated final concentrations (D). The amount of melanin in cell extracts was determined and is expressed relative to the control (A and B). Tyrosinase activity was determined and expressed relative to the control (C and D). Whole cell extracts were analyzed by immunoblotting with an antibody against each protein (E and G). mRNA expression was monitored as described in the legend of Fig. 2 (F and H). Values are given as mean \pm S.D. ($n = 3$). ** $P < 0.01$.

vivo due to the limited amount of EA available). As shown in Fig. 8A, the expression of HSP70 in mouse skin was induced in a dose-dependent manner by the percutaneous administration of EB. The level of expression induced with 1 mg/cm² EB was similar to that observed in transgenic mice expressing HSP70 (Fig. 8A). The induction of HSP70 expression by EB in the skin was also observed at the mRNA level (Fig. 8B). Immunohistochemical analysis also demonstrated that the expression of HSP70 was higher in the epidermis than in the dermis, as described previously [22].

We then examined the effect of EB on UVB-induced epidermal damage. Eighteen hours after the percutaneous administration of EB, mice were subjected to UVB radiation and UVB-induced epidermal damage was estimated by histological observations and measurement of MPO activity, an indicator of the inflammatory infiltration of leukocytes. Histological observations revealed the extensive infiltration of leukocytes and epidermal disruption in skin sections prepared from UVB-irradiated control mice. In mice pre-administered with EB, however, the cutaneous damage was suppressed in a dose-dependent manner (Fig. 8C). MPO activity was increased in control mice in response to the UVB radiation and this activation was suppressed in UVB-irradiated mice pre-treated with EB (Fig. 8D). The level of skin damage and MPO activity in mice pre-treated with 1 mg/cm² EB was similar to that seen in transgenic mice expressing HSP70 (Fig. 8C and D). As described above, HSP70 was reported to suppress the activation of NF- κ B through various mechanisms such as suppression of the inflammatory stimuli-induced degradation of I κ B- α (an inhibitor of NF- κ B) [15]. We therefore examined the effect of EB on the level of I κ B- α *in vivo*. As shown in Fig. 8E and F, UVB irradiation decreased the cutaneous level of I κ B- α , although the level remained significantly higher in mice pre-treated with EB or in transgenic mice expressing HSP70. These results suggest that percutaneously administered EB protects the skin against UVB-induced damage by inducing the expression of HSP70.

Finally, we examined the effect of EB on melanin production in the skin. After the percutaneous administration of EB, mice were exposed to UVB radiation. This cycle was repeated once every 2 days for 8 days and the melanin content was estimated by Fontana-Masson staining of sections or by a narrow-band simple reflectance meter (Mexameter). For this we used the murine tail skin, which resembles human skin in terms of the presence of epidermal melanocytes and UV-dependent melanin production [49]. As shown in Fig. 8G and H, an increase in melanin staining in the basal layer of the epidermis (the dermal/epidermal border) was observed in control mice after UVB irradiation but this increase was significantly suppressed in sections prepared from the mice pre-treated with EB. Measurement of the melanin content with a Mexameter also showed that the content was lower in the UVB- and EB-treated mice than in control mice (mice treated with UVB only) (Fig. 8I). The level of melanin in the skin of mice pre-treated with 1 mg/cm² EB was similar to that seen in transgenic mice expressing HSP70 (Fig. 8G–I). These results suggest that percutaneously administered EB suppresses UVB-induced melanin production by inducing HSP70 expression.

4. Discussion

In this study we purified compounds with HSP70-inducing ability from an ethanol extract of *E. lindleyanum*. Two compounds, EA and EB, were identified, which are optical isomers that have similar pharmacological activities (induction of HSP70 expression, cytoprotection, and inhibition of melanin production). EA and EB were previously identified as major sesquiterpenes in *E. lindleyanum* [33], and although no biological activity was reported for them, a range of biological activities, such anti-tumor, anti-allergic and anti-inflammatory activities have been reported for other

sesquiterpenes [50,51]. This report marks the first time that sesquiterpenes have been described to induce the expression of HSPs. *E. lindleyanum* has been used in China and other Asian countries for the treatment of cough, fever and tracheitis based on its anti-microbial and anti-inflammatory activities [30–32]. Since HSP70 suppresses inflammation through the inhibition of NF- κ B (an inflammation-inducing transcription factor) [13,15], it is reasonable to speculate that the anti-inflammatory effect of *E. lindleyanum* is due to the EA- or EB-mediated induction of HSP70 expression.

A number of compounds have been shown to induce the expression of HSP70; however, in most cases the induced expression is accompanied by a simultaneous decrease in cell viability. These cytotoxic effects of HSP70-inducers may cause side effects, resulting in their unsuccessful development and use in medicines and cosmetics. Therefore, in our previous report where we screened for HSP70-inducers from Chinese herbal extracts [29], we focused our search not only on the capacity of compounds to induce HSP70 expression, but also on their reduced cytotoxic activity. Compared to other Chinese herbal extracts, *E. lindleyanum* extract showed less toxicity at concentrations high enough to induce HSP70 expression; however, as shown in Fig. 1D, at a concentration of 150 μ g/ml, *E. lindleyanum* extract reduced cell viability to 85% and induced a less than 8-fold increase in HSP70 expression. On the other hand, when EA and EB were employed at a concentration of 10 μ g/ml, cell viability remained higher than 98% and HSP70 expression was induced more than 13-fold (Fig. 1D). This could be due to the fact that, in addition to EA and EB, the *E. lindleyanum* extract contains cytotoxic molecules that were separated from EA and EB in the purification process. This low cytotoxicity of EA and EB could be highly advantageous for their future use in medicines and cosmetics.

We showed here on the basis of several different results that the induction of HSP70 expression by EA or EB is mediated by the activation of HSF1. In this way, (i) the induction of expression was also observed for HSPs other than HSP70, (ii) the suppression of HSF1 expression by siRNA inhibited the EA- or EB-mediated induction of HSP70 expression, and (iii) the treatment of cells with EA or EB induced both the phosphorylation and re-localization of HSF1 into the nucleus. Furthermore, EA and EB seem to activate HSF1 by binding to HSP90, thereby inhibiting the interaction between HSP90 and HSF1. This conclusion was arrived at on the basis of the following results: (i) the co-immunoprecipitation of HSP90 with HSF1 was suppressed in extracts prepared from cells treated with EA or EB, and (ii) EB-fixed beads bound to HSP90 but not HSF1, and this binding was suppressed by excess amounts of free EB.

In addition to inducers of HSPs, co-inducers of HSPs (such as bimoclochol and indomethacin) have been paid considerable attention [11]. Co-inducers of HSPs cannot induce the expression of HSPs by themselves, but stimulate the expression achieved by other inducers. Interestingly, some molecules (such as GGA) possess properties of both inducers and co-inducers of HSPs [19,46]. The results of this study suggest that EA and EB may also belong to this category; 1 or 2 μ g/ml EA (or EB) or 3.5% ethanol alone did not induce the expression of HSP70, but when used in combination the EA (or EB) and ethanol clearly induced the expression of HSP70. We also found that treatment of cells with 1 or 2 μ g/ml EA (or EB) likely induced the phosphorylation of HSF1 given the shift in HSF1 protein migration, suggesting that the co-inducer-like actions of these compounds could be due to their capacity to phosphorylate HSF1. A similar mechanism for co-induction has been proposed for indomethacin [52].

With regards to the *in vitro* pharmacological activity of EA and EB, we showed that both exerted cytoprotective effects in addition to an inhibitory effect on melanin production. Pre-treatment of

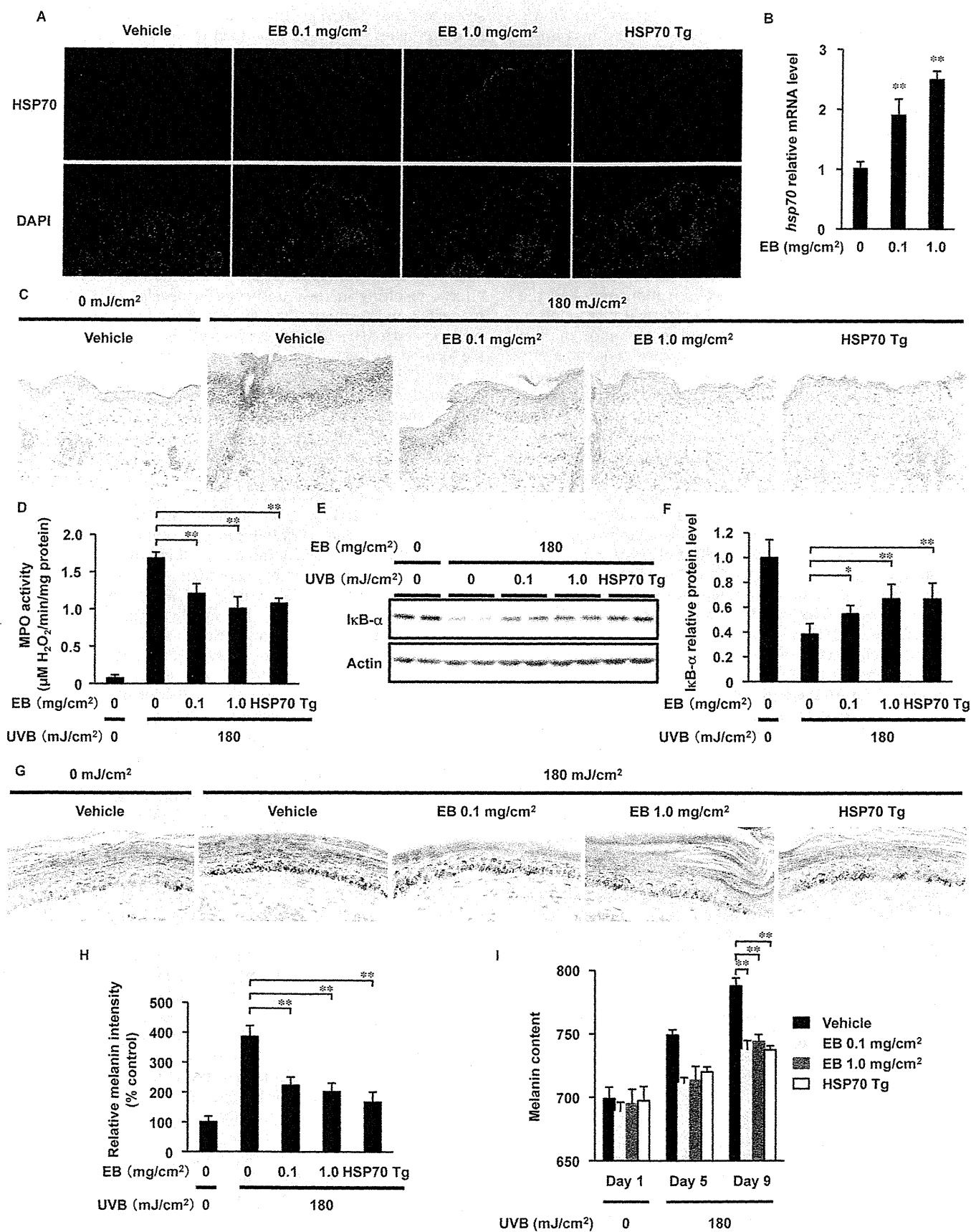


Fig. 8. Effects of EA and EB on UVB-induced skin damage and melanin production *in vivo*. (A–F) The dorsal skin of C57BL/6 (wild-type) mice was percutaneously administered with the indicated doses of EB. Mice were maintained for 18 h (A–F) before sacrifice and the dorsal skin was removed (A and B). Mice were irradiated with indicated doses of UVB (C–F) and sacrificed just prior to dorsal skin removal after 48 h (C, E and F) or 24 h (D). Transgenic mice expressing HSP70 (HSP70 Tg) were treated similarly but without EB administration. Sections of dorsal skin were prepared and subjected to immunohistochemical analysis with an antibody against HSP70 and DAPI staining (A) or hematoxylin and eosin staining (C). The *hsp70* mRNA expression was monitored as described in the legend of Fig. 2 (B). MPO activity was measured as described in Section 2

cells with EA or EB made cells resistant to the induction of apoptosis by subsequent treatment with various stressors (UVB, menadione and heat-shock). In relation to the UVB treatment, we also showed that suppression of HSP70 expression by siRNA suppressed this cytoprotective effect of EA and EB. It has been suggested that HSP70 suppresses a number of steps in the molecular pathways governing apoptosis, including p53 activation, which plays an important role in UVB-induced apoptosis [53,54].

We recently reported that overexpression of HSP70 in B16 cells suppresses IBMX-induced melanin production and increases the activity and expression of tyrosinase, but does not affect the expression of MITF. As a mechanism to explain this phenomenon, we showed that HSP70 physically interacts with MITF and suppresses MITF's transcription activity on the tyrosinase gene [28]. To this extent, we showed here that EA and EB decrease the activity and expression of tyrosinase without decreasing the expression of MITF in IBMX-treated cells.

As for *in vivo* relevance of these *in vitro* results, we showed that the percutaneous administration of EB increased HSP70 expression in the skin in a dose-dependent manner. Although we did not show the penetration of EB into epidermis, the result suggests that EB could reach the region and induce HSP70 expression. We also suggest that the percutaneous administration of EB suppresses UVB-induced skin damage, inflammatory responses and melanin production by inducing HSP70 expression *in vivo*. Since the use of hypopigmenting reagents could be useful as drugs and cosmetics, a number of compounds that inhibit tyrosinase and/or MITF have been discovered; however, most of their cosmetic and pharmaceutical applications have not been successful due to skin irritation side effects [55]. This seems to be due to the fact that UV-induced melanogenesis plays a protective role against UV-induced skin damage. Based on the results of this study, we propose that EA or EB could be cosmetically and pharmaceutically beneficial because HSP70 protects cells from UV-induced damage in conjunction with inhibition of melanin production.

An ameliorative effect of HSP70 due to its cytoprotective, anti-inflammatory and molecular chaperone (quality control of proteins) properties has been reported for various diseases. For example, we have shown using transgenic mice and GGA that HSP70 protects against irritant-produced lesions in the stomach and small intestine, as well as in inflammatory bowel disease-related experimental colitis, pulmonary fibrosis and in Alzheimer's disease-related phenomena in mice [18,20,35,56–58]. EA or EB could thus be therapeutically beneficial for use in these diseases.

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(D). Whole cell extracts were analyzed by immunoblotting with an antibody against I κ B- α or actin (E). The intensity of the I κ B- α band relative to the actin band is shown (F). Values are given as mean \pm S.E.M. ($n = 5-6$). ** $P < 0.01$. (G-I) The tail skin of C57BL/6 (wild-type) mice was percutaneously administered with indicated doses of EB and, after 18 h, mice were irradiated with 180 mJ/cm² UVB. This cycle was repeated once every 2 days for 8 days (i.e., a total of 4 times, on days 2, 4, 6, 8). Transgenic mice expressing HSP70 (HSP70 Tg) were treated similarly but without EB administration. Sections were prepared from the tail skin on day 9 and subjected to Fontana-Masson staining (G). The intensity of epidermal melanin in each section was determined and is shown (H). The amount of melanin in the tail skin was also measured by Mexameter (day 1, 5, 9) (I). Values are given as the mean \pm S.E.M. ($n = 9-10$). ** $P < 0.01$.

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