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ORIGINAL PAPER

Inhibition of melanogenesis by the extract from *Agaricus blazei* without affecting iNOS gene expression

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Abstract Tyrosinase is a key enzyme in melanin synthesis. Owing to enlargement of availability of edible types of mushrooms in food medicines, we investigated effects of *Agaricus blazei* (ABE) on tyrosinase activity using L-tyrosine and L-3, 4-dihydroxyphenylalanine (L-DOPA) as the substrate in normal human epidermal melanocytes (NHEM). ABE inhibited tyrosinase activity similar to arbutin and Vitamin C as two whitening agents in a dose dependent manner. In agreement with this, treatment of the cells with ABE (3–100 μg/ml) reduced melanin content up to 57% of the control in NHEM. In addition, production of nitric oxide (NO) which has the ability of inducing tyrosinase activity in melanocytes was also suppressed by ABE treatment. Furthermore, ABE inhibited NO production in lipopolysaccharides (LPS)-stimulated RAW264.7 macrophages, in a dose dependent manner but without affecting iNOS mRNA expression indicated by reverse transcription-PCR (RT-PCR) technique. These findings suggest that ABE inhibits melanin production via partial inhibition of tyrosinase activity and NO production. This hypopigmenting effect of water soluble *Agaricus blazei* extract could be useful for the treatment of some skin disorders.

Keywords *Agaricus blazei* · Melanin · Skin whitening · Melanocytes · RAW264.7 cells, RT-PCR

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Introduction

Tyrosinase (polyphenol oxidase) plays rate limiting roles in the production of melanin by melanocytes. Melanin pigment, responsible for visible skin color, is formed through a series of oxidative reactions involving the amino acid, tyrosine. Tyrosinase catalyses three main steps in melanogenesis; the hydroxylation of L-tyrosine to L-dihydroxyphenyl alanine (l-DOPA), the oxidation of L-DOPA to DOPAquinone, and the additional oxidation of 5, 6 dihydroxyindole to indol-quinone (Hearing and Tsukamoto 1991). Therefore, almost all factors affecting melanin production exert their action either directly or indirectly via stimulation of tyrosinase (Busca and Ballotti 2000).

Tyrosinase catalyzes both constitutive and UV-induced melanogenesis. Ultraviolet (UV) radiations are well known for their role in melanogenesis. Skin exposure to UV radiation of solar light releases excessive reactive species such as nitric oxide (NO) that is usually linked to skin disorders (Roméro-Graillet et al. 1997; Kim et al. 2006). After UV exposure, tyrosinase activation and upregulation of gene expression by NO released from keratinocytes induce melanogenesis (Roméro-Graillet et al. 1997; Sasaki et al. 2000). Anti-oxidants and NO scavengers such as vitamin C down-regulate UV-induced melanogenesis (Silva and Maia Campos 2000; Xiao et al. 2007). In agreement with this, melanogenesis is inhibited by NO

scavengers such as ascorbic acid and tyrosinase is upregulated by NO (Maeda and Fukuda 1996; Quevedo et al. 2000; Silva and Maia Campos 2000; Xiao et al. 2007). Furthermore, many tyrosinase inhibitors and NO scavengers are useful in cosmetics as skin-whitening agents and also as remedies for pigmentation disturbances (Wang and Hebert 2006; Ando et al. 2007).

Recently, a great deal of interest has been focused on deriving tyrosinase inhibitors from plant origin. In fact, anti-melanogenesis activities of several herbal medicines have been evaluated by their capabilities to inhibit tyrosinase (Zhong et al. 2006; Baek et al. 2008; Momtaz et al. 2008). It has been reported that *Agaricus blazei* has wide range of pharmacological actions (Wasser and Weis 1999; Lavi et al. 2006; Yuminamochi et al. 2007). For example, *A. blazei* has benefits on health through their anti-neoplastic (Mizuno et al. 1998; Kaneno et al. 2004), antimutagenic (Menoli et al. 2001), and antiviral activities (Sorimachi et al. 2001). Although *A. blazei* is rich in tyrosinase that is responsible for mushroom browning, this study was carried out to obtain direct evidence for the hypopigmenting effect water soluble *A. blazei* extract through its action on mammalian tyrosinase. First, we evaluated the effect of ABE on melanin content in normal human epidermal melanocytes (NHEM). Next we investigated the effect of ABE on tyrosinase activity in NHEM and NO production in RAW264.7 cell line. Our results showed that the components of *A. blazei* extract (ABE) had an ability to inhibit melanogenesis by suppressing tyrosinase activity and NO production.

Materials and methods

Reagents

L-ascorbic acid (Vitamin C, Merck, Darmstadt, Germany), Dulbecco's modification of Eagle's Medium (DMEM) supplemented with glutamine was purchased from ICN Biomedicals (Aurora, OH, USA), all remaining reagents were obtained from Sigma (Louis, MO, USA). All reagents used in reverse transcription-PCR were purchased from Invitrogen (Carlsbad, CA, USA).

Preparation of ABE

Agaricus blazei fruits were isolated in Assiut governorate and identified in Department of Botany, Faculty of Science, Assiut University, Egypt. *A. blazei* is an edible plant in Egypt. Water soluble ABE was extracted according to the method of Mizuno et al. (1998) with slight modifications. In brief, the dried fruiting bodies of *A. blazei* were extracted with boiled water. Then, these extracts were centrifuged at 1,800*g* for 10 min to remove solid particles, freeze dried. One gram of ABE powder was dissolved in 100 ml of phosphate-buffered saline (PBS) just before use in each experiment.

Cell culture and incubation with reagents

Normal human epidermal melanocytes (NHEM) are primary cells derived from epidermis of normal human skin, bought from Cell Applications (INC. USA). NHEM were cultured in complete Melanocyte Growth Medium prepared by adding growth supplements (135-GS1, GS2 and GS3) to Basal Medium as indicated in manufacturer protocol. After the cells had become almost confluent, they were further cultured in a medium containing ABE, and incubated with reagents at 37°C in CO_2 incubator. ABE (3, 10, 30, and 100 μg/ml) was added in the presence or absence of 100 μM of sodium nitroprusside (SNP) as NO source. The viability of the cells was examined by using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma), which is based on the ability of a mitochondrial enzyme, succinate dehydrogenase, to cleave MTT to the blue compound formazan as previously described (Mahmoud et al. 2010). Ethics and experimental procedures were approved by Sohag University Animal Care and Use Committee.

Determination of melanin content in melanocytes

Determination of melanin content was performed as in published protocol (Curto et al. 1999) with some modifications. NHEM were cultured at in 6-well plates, after 24 h, the cells were treated with ranging concentrations of ABE (3, 10, 30, 100 μg/ml medium). The cells were trypsin harvested and washed 3 times with PBS. Then 1.0×10^7 cells were air-dried and dissolved in 200 μl of 1 N NaOH containing 10% dimethyl sulfoxide (DMSO). Then lysates were heated in water bath at 80°C for 1 h and cooled; the amount of melanin was determined spectrophotometrically based on absorbance of at 475 nm.

Assays of tyrosinase activity

DOPA oxidase activity in NHEM cellular extract was assayed according to the method of Maeda and Fukuda (1996). Cells were cultured in 6-well plates. The confluent cells were treated with ranging concentrations of ABE; 3, 10, 30, and 100 μg/ml medium for 48 h. Cells were harvested by trypsinzation and collected by centrifugation at 1,000*g* for 5 min, washed 3 times with PBS and centrifuged at 1,000*g* for 5 min. About 1.0×10^7 cells were lysed in 1 ml PBS containing 0.5% Triton X-100. After sonication in ice (20 s), cell extracts were clarified by centrifugation at 10,000*g* for 15 min at 4°C. Reaction mixture (3 ml, freshly prepared)

composed of cell lysate, 0.1% L-DOPA, and 0.1 M PBS (pH 6.8) were kept in ice. The mixture was then incubated at 37°C for 1 h for assay of DOPA chrome by spectrophotometry at 475 nm. DOPA chrome amount/cell, was expressed as a percent of control (=100%) inhibition. Auto-oxidation of L-DOPA was corrected in controls.

NO donor

To investigate the effect of ABE on NO-induced tyrosinase activity, donor sodium nitroprusside (SNP) was used. The direct NO scavenging assay was carried out according to Roméro-Graillet et al. (1997) with some modifications. Briefly, 10 µl of SNP (100 µM) solution were incubated either alone, with ascorbic acid (500 µM) as positive control or in combination with different concentrations of ABE (3, 10, 30, and 100 µg/ml) for 48 h.

The nitrite levels of the mixture were then determined by Griess assay. NO scavenging activity was calculated a percent of control (=100%) inhibition.

Murine monocyte/macrophage RAW264.7 cells culture

RAW264.7 cell line was suspended in DMEM containing 10% fetal calf serum (FCS), 200 µg/ml gentamycin and 100 U/ml penicillin. Cell counts were made using trypan blue exclusion stain. Non-adherent cells were washed away with warm DMEM. Adherent cells were re-suspended in serum-free medium and re-incubated at 37°C and 5% CO_2 for 24 h with the reagents. A 6-well culture plate contains 1.5×10^6 cells/ml was further incubated with reagents at 37°C in CO_2 incubator. Lipopolysaccharides (LPS) (5 µg/ml) was incubated in the presence or absence of ABE (3, 10, 30, and 100 µg/ml). After 6 h incubation, RT-PCR was performed on the cells and after 24 h, culture supernatants were collected for nitrite assay.

Determination of nitrite level

In RAW264.7 cell line, NO produced was assessed by measuring its stable degradation product, nitrite (NO_{2-}), as described previously (Green et al. 1982). The culture supernatants (50 µl) were mixed with equal volume of Griess Reagent (modified). Absorbance of the samples was read at 540 nm by micro-plate reader. The concentrations of nitrite in supernatant were calculated from the standard curve of sodium nitrite.

RNA isolation and reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from RAW264.7 cells homogenates according to single step method of Chomczynski and Sacchi (1987) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm, and its purity was evaluated by the ratio of absorbance at 260/280 nm (>1.8). iNOS mRNA was assessed by RT-PCR. After digestion of genomic DNA by treatment with deoxyribonuclease I (Invitrogen), first-strand cDNA was synthesized from 2 µg of total RNA by using Superscript II RNase H-reverse transcriptase and oligo (dT) primer (Invitrogen). For performing PCR, 1 µl of template cDNA was added to a PCR cocktail consisting of 10 mM Tris–HCl, 50 mM KCl, 1.25 mM $MgCl_2$, 10 mM deoxy-NTPs, 200 nM primers, and 0.05 U platinum Taq DNA polymerase (Invitrogen) for a final volume of 10 µl. The sense and antisense primers for the housekeeping β-actin gene were 5′-TAG CCC TGA AGT ACC CCA TTG-3′and 5′-TCA GGA TCT TCA TGA GGT AG-3′, respectively (predicted size = 434 bp) and those for iNOS were 5′-TAG AAA CAA CAG GAA CCT AC-3′ and 5′-AAC ATC TCC TGG TGG AAC A-3′, (predicted size = 955 bp) respectively. All primers were purchased from Invitrogen. PCR was performed with a thermal cycler (GeneAmp. PCR system 2700, Applied Biosystems, Foster city, CA, USA). The amplifications were performed by 20 cycles or 25 cycles for β-actin or iNOS, respectively. The PCR products were analyzed in a 1% 0.5 × TBE agarose gels electrophoresis and stained with ethidium bromide (0.2 µg/ml). The gels were exposed to UV light with a UV transiluminator (UVP Laboratory Products, Upland, CA, USA). The amplified DNA bands were analyzed by using the image analysis software ScionImage (Scion Corp., Frederick, MD, USA).

Statistical analysis

Each experiment was repeated several times and performed in duplicates. Data were analyzed by one-way ANOVA, and significant differences were compared by using Tukey's Studentized Range Test. Differences were significant when $P < 0.05$.

Results

Effect of ABE on melanin content

Since NHEM constitutively produce melanin provided that complete growth medium with supplements are used. Arbutin 20 µg/ml (as a positive control) was added to confluent cells. The reduction in melanin content was expressed as a percentage of that of non-treated cells. After 48 h, maximum reduction in melanin production (42.0 ± 3%) was obtained by arbutin and followed by dose dependent concentrations of ABE (Fig. 1). Maximum

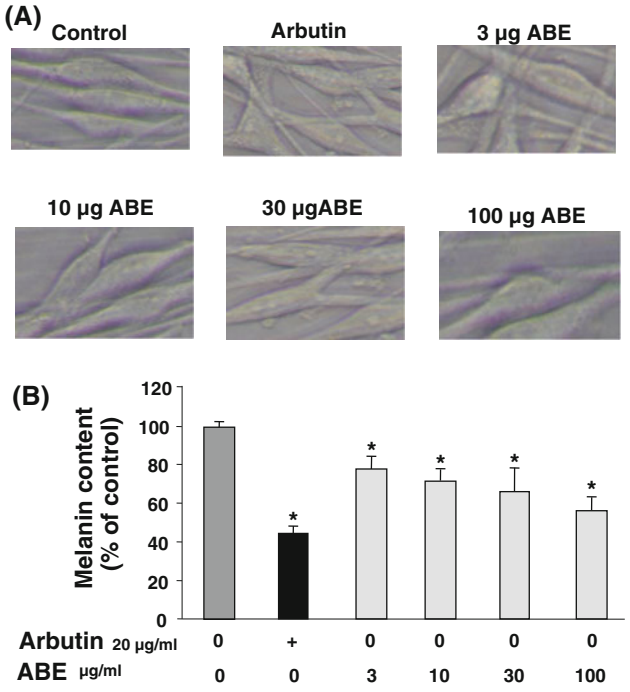

Fig. 1 **a** Morphological appearance of non-treated NHEM cultured in minimal medium, cells were treated with arbutin or ABE (3, 10, 30, and 100 µg/ml) under phase contrast microscopy (400×). **b** Effect of ABE on melanogenesis in NHEM. ABE was treated for 48 h and melanin produced by NHEM was expressed as % of control inhibition. Values were measured as mean ± SD in duplicate, and arbutin was used as positive control

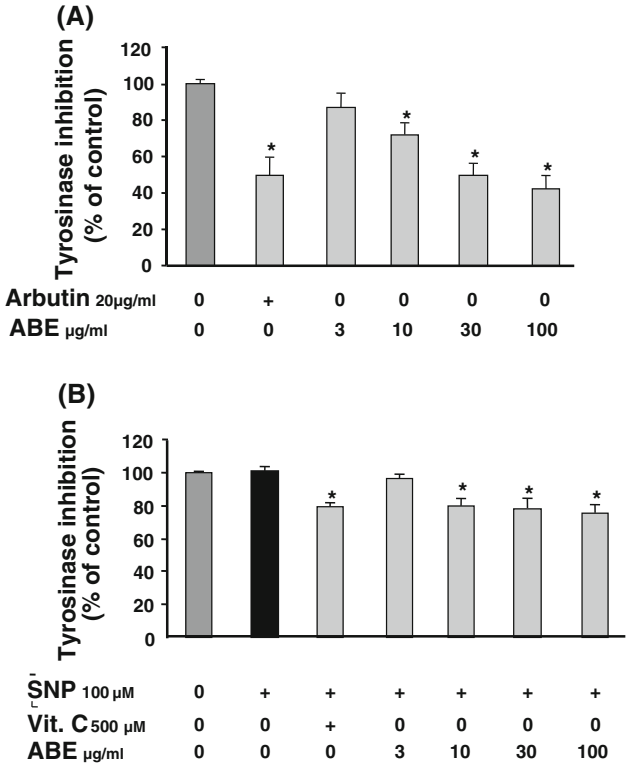

Fig. 2 **a** Effect of ABE treatment for 48 h on melanin produced by NHEM expressed as % of control inhibition in the presence of NO donor and Vit. C used as whitening agent. Data are presented as mean ± SD (in duplicate). **b** Effect of NO on melanocyte melanogenesis. **a** Effect of NO donors on tyrosinase activity and melanin synthesis of melanocytes. Melanocytes were exposed to SNP (100 µM) for 48 h. Results are expressed as a percentage of basal activity from control melanocytes inhibition. Each value represents the mean ± SE of triplicates of one representative experiment

inhibition effect of ABE on melanin production was about 57% at 100 µg/ml culture medium. After 5-days treatment with ABE (100 µg/ml) melanin produced by NHEM was similar to positive control arbutin (data not shown).

Effect of ABE on tyrosinase activity

In addition to arbutin 20 µg/ml, as positive control, in separate wells ABE was incubated with NHEM for 48 h. In vitro assay for tyrosinase activity showed significant reduction at 10, 30, and 100 µg/ml of ABE (expressed as % of control inhibition, Fig. 2a). Melanocytes per se never produce NO even after exposure to UV radiations but neighboring epidermal cells did so. Moreover, UV-stimulation of melanogenesis can be mimicked by exogenous NO donors. Therfore, freshly prepared sodium nitroprusside (SNP) 100 µM was used. Fourty eight-h-old solutions of NO donors (which only contain the inactive by-products) were used as control. In addition, ABE (10, 30 and 100 µg/ml) inhibited tyrosinase activity in the presence of NO source (Fig. 2b). NHEM was treated with NO donor (100 µM SNP) to mimic the effect of exposure to UV radiation. L-ascorbic acid 500 µM was used as scavenging agent to overnight incubation with NO donor; our data showed that 48 h treatment of NHEM with 10, 30 and 100 µg/ml ABE was more effective than L-ascorbic acid (Fig. 2b).

Effect of ABE on NO production in cultured murine macrophages

The present work investigated the effects of ABE on NO production induced after treatment of RAW264.7 macrophages/monocyte cells (1.0×10^6) with LPS (5 µg/ml). Drug treatment under described concentrations showed no effects on RAW264.6 cell viability. Non-treated cells (without LPS stimulation), produced minimal levels of nitrite (6.1 ± 0.7 µmol/10^6). However, after incubation with LPS for 24 h, nitrite production was markedly increased to 43.1 ± 3.6 and 57 ± 4.2 (µmol/10^6) as shown in Fig. 3a. Nitrite production was reduced by ABE treatment in dose dependent concentrations. Although this inhibitory effect of ABE on nitrite production, iNOS mRNA expression was not affected in LPS/ABE treated wells as shown in RT-PCR gel image (Fig. 3b).

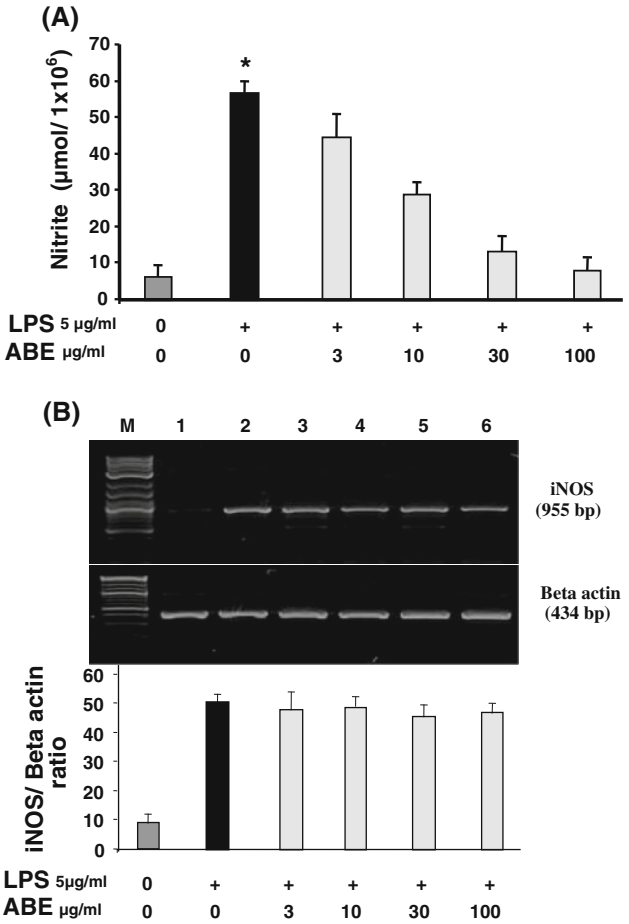

Fig. 3 **a** Effect of ABE Treatment on nitrite level in LPS stimulated RAW 264.7 cell line. Data are presented as mean ± SD. **b** Gel electrophoresis of RT-PCR for determination of effects ABE on iNOS/beta actin mRNA expression in LPS stimulated RAW 264.7 cell line. Cells were incubated with the medium alone for 6 h (*lane 1*) and with the medium followed by LPS (µg/m) for 6 h (*lanes 2*, *3*, *4*, *5* and *6*) and followed by ABE in gradient concentrations as shown in *lanes* (*3*, *4*, *5* and *6*) for 6 h. RNA was extracted and analyzed by RT-PCR for iNOS and *β*-actin mRNA expression. The data shown are representative of four experiments (mean ± SD) and are expressed in relative units, which were determined by the ratio of iNOS to *β*-actin mRNA at each time point

Discussion

Melanogenesis is exquisitely controlled by tyrosinase. Inhibiting tyrosinase and avoiding UV exposure can reduce melanin synthesis. Tyrosinase inhibitors are thought to be the most useful way for treating pigmentation disorders (Maeda and Fukuda 1996). In the present study, the inhibitory effects of ABE on melanin content and tyrosinase activity were firstly evaluated. Next, tyrosinase activity and melanin content were compared with NHEM treated with arbutin. ABE inhibited tyrosinase after adding NO donor to NHEM. Finally, ABE also inhibited NO production in LPS-stimulated RAW264.7 cells. These results suggest that ABE inhibits melanin synthesis via suppressing tyrosinase activity and NO production.

Although *A. blazei* fungus is rich in tyrosinase that are responsible for its browning. It seems reasonable to assume that *A. blazei* enzyme tyrosinase is inactivated after boiling. However, *A. blazei* contains substrate for tyrosinase that could compete on the enzyme active sites (Jolivet et al. 1999). A plausible candidate would be a phenolic amino acid termed gamma-glutaminyl 1-3, 4-dihydroxybenzene (GHDB). This metabolite seems to be the melanin precursor for mushroom browning, and it is a substrate for tyrosinase (Oka et al. 1981). It could be possible that GHDB or some derivative after extract boiling could affect the tyrosinase assay and the formation of dopa-melanin polymers. *A. blazei* has wide range of biological activities, as contains multiple pharmacologically active compounds, because its extracts induce a wide range of actions in vivo and in vitro (Menoli et al. 2001; Ahn et al. 2004; Kimura et al. 2004). These data suggests actions for both water soluble (Ito et al. 1997) and water insoluble extracts (Kawagishi et al. 1989). In the present study, water soluble extract of *A. blazei* reduced the melanin content in NHEM. Considering the fact that NHEM synthesize melanin in complete growth media, thus it is rational to assume that ABE inhibited melanin synthesis.

To our knowledge, this is the first demonstration of a direct action of ABE on melanocytes. It is generally accepted that the increase of melanin production in melanocytes is mediated by tyrosinase; its activity is enhanced following stimulation. In support of this view, the application of arbutin, a competitive tyrosinase inhibitor, reduced melanin production in cultured NHEM, as it is reported by Maeda and Fukuda (1996). Arbutin, a b-D-glucopyranoside derivative of hydroquinone, also inhibits tyrosinase activity competitively but at non-cytotoxic concentrations in cultured melanocytes (Maeda and Fukuda 1996). Vitamin C, as a free radical scavenger, has been used as hypopigmenting agent due to its anti-oxidant ability to inhibit oxidative stress promoted melanogenesis in normal human epidermis melanocytes (Silva and Maia Campos 2000; Xiao et al. 2007). In present study Vitamin C has been used as No scavenger in NHEM. The inhibition of tyrosinase activity may not be related to a non-specific toxicity of SNP, because NHEM vitality determined by MTT assay was not affected under our experimental conditions (100 µM of SNP for 48 h). In accordance with this, Roméro-Graillet et al. (1997) reported that several specific effects of SNP on human melanocytes can be observed without accompanying cytotoxicity at a higher concentration of SNP (200 µM) and longer incubation (96 h).

After the addition of NO donor to NHEM, ABE also inhibited tryrosinase activity that was similarly inhibited by L-ascorbic acid (Fig. 2a). These results suggest that

tyrosinase activation by NO is also affected by ABE. This notion is further supported by the finding that ABE decreased NO production in LPS-stimulated RAW264.7 cells (Fig. 3a). The present study shows that ABE has inhibitory roles on both melanogenesis and NO production. Interestingly, ABE (100 μg/ml) was found to have a more inhibitory effect than arbutin. In fact, L-tyrosine constitutes the first substrate in the melanin synthesis cascade controlled by tyrosinase. However, mechanism of ABE inhibition to tyrosinase is still unknown. One possibility is that extracts of *A. blazei* has phenolic compouns such as the well known phenloic amino acid GHDB. Many investigators (Kim et al. 2005, 2006) have reported that a number of naturally occurring melanogenic inhibitors contain a phenol structure (Monach et al. 2004; No et al. 2004; Kim et al. 2005; Kim 2007) that could potentially elicit competitive inhibition of tyrosinase as does arbutin (Nakamura et al. 2003; Ando et al. 2007).

It is known that NO donors are a potent stimulator for tyrosinase and melanin neosynthesis in human melanocytes (Roméro-Graillet et al. 1997). ABE could also directly regulate melanogenesis. This action of ABE is difficult to interpret at present. However, in this part, it could be similar to L-ascorbic acid. Hence, L-ascorbic acid has well known ability to scavenge free radicals, and affects one step in melanin synthesis; hydroxylation step of L-tyrosine (Sasaki et al. 2000). This anti-oxidant property of ABE in terms of suppressing NO production could be involved in reduction–oxidation reactions-activated tyrosinase within melanogenic signaling pathway.

In this study, exposure of RAW264.7 macrophages to LPS was associated with an accumulation of nitrite in the medium, suggesting that it enhanced NO production. In addition, the LPS-induced NO production was inhibited by treatment with ABE. Since, RT-PCR analysis indicated that the gene expression of iNOS in RAW264.7 cells that had been treated with LPS was not affected by ABE. Therefore, these findings indicated that the inhibition of LPS induced NO production by ABE occulted independently to iNOS gene expression. However, it can be concluded that ABE inhibited NO production through a mechanism upstream to iNOS mRNA level. The key molecules on which ABE acts remain to be elucidated.

Conclusions

In conclusion, our results demonstrate that ABE has hypopigmenting effects in NHEM. We found that ABE is a potent inhibitor to melanin synthesis, and that these inhibitory effects are caused by inhibition of enzymes tyrosinase and iNOS activities in terms of oxidation of L-DOPA and NO production. RT-PCR results indicated that the gene expression of iNOS in treated RAW264.7 cells with LPS was not affected by ABE. The results might suggest potential uses of ABE as an effective skin-whitening agent.

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