Nitric oxide enhances melanogenesis of alpaca skin melanocytes in vitro by activating the MITF phosphorylation

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Abstract Ultraviolet (UV) B radiation can cause skin-tanning via the synthesis of melanin which is synthesized by specific tyrosinase and tyrosinase-related enzymes expressed in melanocytes. It is reported that several melanogenic factors are released from keratinocytes and other cells surrounding melanocytes in the skin following UV radiation. Some of them are reported to up-regulate tyrosinase gene expression through a different pathway, but most regulate tyrosinase via microphthalmia-associated transcription factor (MITF). It is unknown whether an NO-induced pathway regulates melanogenesis via MITF in vitro. In this study, we investigated this problem because it is important for our understanding of how to enhance the coat color of alpaca. We set up three groups for experiments using alpaca melanocytes: the control cultures were allowed a total of 5 days growth; the UV group cultures were also allowed 5 days of growth like the control group, but were then irradiated once everyday with 312 mJ/cm² of UVB; the UV + L-NAME group was the same as the UV group, but with the addition of 300 µM L-NAME every 6 h. To determine the NO inhibition effect, NO product was measured. To determine the effect of NO on MITF, the expression levels of the MITF gene and protein were measured by immunofluorescence, quantitative real-time PCR and western immunoblotting. To determine the influence of NO on MITF phosphorylation, phosphorylated MITF protein (p-MITF) was measured by western immunoblotting. To determine the effect of NO on melanogenesis, the melanin content was measured. The results provide exciting new evidence that NO can enhance melanogenesis in alpaca skin melanocytes by stimulating MITF phosphorylation.

Keywords Nitric oxide · Alpaca · Melanocyte · Microphthalmia-associated transcription factor · Melanogenesis

Introduction

Ultraviolet (UV) B radiation can cause skin-tanning via the synthesis of melanin, which is synthesized by specific tyrosinase and tyrosinase-related enzymes expressed in melanocytes. It is reported that several melanogenic factors are released from keratinocytes and other cells surrounding melanocytes in the skin following UV radiation. Relevant melanogenic factors [1] include nitric oxide (NO), adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), endothelin-1 (ET-1), prostaglandins, histamine, and thymidine dinucleotide [2]. Some of these factors are reported to up-regulate tyrosinase gene expression through different pathways, but most pathways regulate tyrosinase via microphthalmia-associated transcription factor (MITF).

The transcription factor MITF is a key regulator of melanocyte development, function, and survival by
regulating genes involved in the cell cycle and melanocyte differentiation [3, 4]. MITF acts as a master regulator of pigment production through activation by α-MSH, agouti signaling protein, and Kit ligand [3, 5]. Then, activated pigmentation transcription MITF [6] increases levels of the major pigmentation proteins including tyrosinase (TYR), dopachrome tautomerase (DCT), and tyrosinase-related protein-1 (TYRP1) [7], as well as dendricity [8], pigment production [7, 9, 10], and cell growth [7, 10, 11].

NO is considered to play an important role in UV-induced hyperpigmentation [12, 13]. NO is generated from l-arginine by the catalytic action of three NO synthase (NOS) isoforms (neuronal nNOS or NOS1, inducible iNOS or NOS2, and endothelial eNOS or NOS3) [14–16]. The downstream targets of NO in melanocytes remain to be fully elucidated. It is likely that NO plays a central role in the activation of tyrosinase via guanylate cyclase and PKG activation [12]. NO may also be involved in the induction of the tyrosinase mRNA message, which is induced within 2 h of in vitro application of an NO donor [13]. Other NO-activated pathways, however, may also be important. In the presence of oxygen, NO can react with the melanin-related metabolites 5,6-dihydroxyindole and its 2-carboxylic acid (DHICA) in cell free systems resulting in the deposition of melanin-like pigments [17]. Evidence from other cell systems suggests that NO can suppress inositol triphosphate (IP3) synthesis [18] and the release of Ca²⁺ via modulation of the IP3 receptor [19, 20]. The enhancement of tyrosinase gene expression via the cyclic guanosine 3′,5′-monophosphate(cGMP) pathway may be a primary mechanism for NO-induced melanogenesis[13].

However, it is not known whether an NO-induced pathway via MITF enhances melanogenesis in vitro. This is important for our understanding of how to enhance the coat color of alpaca.

NO is released by melanocytes under UV radiation, but other melanogenic factors are released from keratinocytes and other cells surrounding melanocytes in the skin. In this study, we used cultured alpaca skin melanocyte under UV radiation conditions to investigate the effect of the presence or absence of NO (using N⁵-nitro-l-arginine methyl ester hydrochloride, L-NAME, a specific NOS inhibitor) on MITF and melanogenesis. This can help us to understand the molecular and signaling mechanisms of NO-induced melanogenesis.

Materials and methods

Melanocyte culture

All alpaca melanocyte cell cultures used in this study were established in the Laboratories of Alpaca Biology, College of Animal Science and Technology, Shanxi Agricultural University, China [21]. Melanocyte cultures were maintained in Melanocyte Basal Medium supplemented with 0.2 µg/ml cholera toxin (Sigma), 0.05 mg/ml gentamicin, 2.5 µg/ml fungizone, 50 µg/ml bovine pituitary extract (BPE), 0.5 µg/ml hydrocortisone, 1 ng/ml bFGF, 5 µg/ml insulin, and 10 ng/ml TPA. They are primary cells, so we used the third passage number for considerations in terms of passage numbers to the outcomes.

Experimental design

We set up three groups for the experiments. The control and cultures were allowed a total of 5 days of growth in 6 cm dishes; the UV group was treated like the control group, but the melanocytes were then irradiated once every day with 312 mJ/cm² of UVB from an SE lamp (FL 20 SE40 lamp, Toshiba, Tokyo, Japan); the UV + L-NAME group was treated the same as the UV group but with the addition of 300 µM L-NAME every 6 h. The dose of UVB utilized was shown in previous studies [22] to effectively induce mRNA and protein in cultured alpaca melanocytes for genes controlling pigmentation [22]. After 5 days, the samples were harvested [22]. Using the three groups, three experimental protocols were designed. Protocol A was designed for identifying the inhibitory effect of L-NAME on NO by measuring the concentration of nitric oxide in the culture medium. In Protocol B, the expression level of MITF in melanocytes was assessed to ascertain whether NO can affect the expression of MITF. Indirect immunofluorescence microscopy was performed to identify the MITF protein, quantitative real-time PCR was used to determine the expression level of the MITF gene, and western blot analysis was used to determine protein expression level of MITF and MITF phosphorylation. Protocol C assessed the effect of NO on induced melanin synthesis by checking the melanin content.

Nitric oxide assay

The amount of stable nitrite (nitrite and nitrate), the end products of NO generation by melanocytes, was determined using the nitric oxide assay kit (NJCBio, Nanjing, China) according to the manufacturer’s instructions (n = 5 in each group).

Immunofluorescence

Cells were washed in PBS, fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized using 0.1% Triton X-100 for 2 min, and non-specific sites blocked using 2% bovine serum albumin (BSA) for 30 min. Anti-MITF (C17, 1:200,
Santa Cruz Biotechnology, Santa Cruz, CA) was applied for 1 h at room temperature (RT) in 2% BSA and visualized with a 1:500 FITC-conjugated goat anti-rabbit secondary antibody (clone 81-6111, Zymed Laboratories, Inc., San Francisco, CA). Specific staining was identified by comparison with negative controls that were processed with phosphate buffered saline (PBS).

RNA preparation and quantitative real-time PCR

To confirm the mRNA expression levels of MITF, quantitative real-time PCR was performed. Total RNA was obtained from melanocytes using Trizol reagent (Invitrogen, Carlsbad, CA USA) according to the manufacturer’s instructions (n = 3 in each group). The reverse transcription reaction was performed using 1 μg of total RNA and the RT-for-PCR kit (Takara, Dalian, China) according to the manufacturer’s instructions. The levels of mRNA were measured by the quantitative real-time PCR method using SYBR® Premix Ex Taq™ II (Takara, Dalian, China) and the Agilent MX3005P QPCR System according to the manufacturer’s instructions. We designed MITF and 18S rRNA (endogenous control gene) primer as shown in Table 1. The conditions for real-time PCR were as follows: preheating at 95°C for 10 s, followed by 40 cycles of shuttle heating at 95°C for 15 s, 55°C for 25 s, and 72°C for 10 s. All mRNA levels from each treatment were normalized to the corresponding amount of 18S rRNA. All samples were run in triplicate. Quantitation of gene expression by real-time PCR was evaluated using the Comparative CT Method according to the manufacturer’s guidelines. The analysis of the relative quantization required calculations based on the CT as follows:

1. ΔCT, the difference between the mean CT values of the samples evaluated with target gene specific primers and those of the same samples evaluated with 18S rRNA specific primers.
2. ΔΔCT, the difference between the ΔCT values of the samples and the ΔCT value of the calibrator sample (un-treated control).
3. 2^−ΔΔCT, which yields the relative mRNA units representing the fold induction over the control.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequence of primers and PCR amplicon size of target gene</th>
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<tr>
<td>Target gene</td>
<td>Sequence primers (5′ → 3′)</td>
</tr>
<tr>
<td>MITF</td>
<td>F:CGAAAGTTGCAAACGRGAACAGCA</td>
</tr>
<tr>
<td></td>
<td>R:GAGCCTGCAATTCAAGTTCCGTGA</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F:GAAGGGACACCAAGGAGGAT</td>
</tr>
<tr>
<td></td>
<td>R:CAGACAAATCACTCCACCA</td>
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Cell extraction and western blotting

After treatment, the 6-cm dishes were placed on ice, rinsed with ice-cold PBS and 400 μl of cell disruption buffer (RIPA Lysis Buffer, Beyotime, Shanghai, China) and 1 mmol/l PMSF were added and sonicated for 10 s on ice. Cell lysates were centrifuged at 14,000×g at 4°C for 5 min. Protein concentrations were measured by the BCA method using bovine serum albumin as the standard. Extracts were heat denatured at 95°C for 5 min, and equal amounts (35 μg/lane) of protein from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. Western immunoblotting was carried out. Primary antibodies were used at the following dilutions: anti-MITF (C17, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-MITF (1:500, Bioworld, Shanghai, China), and anti-β-actin (1:1000, CWBIO, Beijing, China). Dilutions were prepared in fresh 5% skimmed milk blocking buffer, and incubations were performed overnight at 4°C. After washing in TBS with 0.1% Tween 20, the appropriate horseradish peroxidase conjugated secondary antibody (Zymed Laboratories, Inc., San Francisco, CA) was incubated with the blot for 1 h at RT (1:5000 in 3% blocking buffer). Following washing, the blot was exposed to Supersignal West Pico (TIANGEN, Beijing, China). All experiments were performed in triplicate.

Melanin content

Constitutive and UVB-induced melanin contents were compared on the fifth day after plating. Melanin content was determined as described by Lee et al. [23]. Melanocytes were harvested, rinsed with PBS, and counted. Melanin was solubilized in 0.2 mol/l NaOH (10⁶ cells/ml) and measured spectrophotometrically at an absorbance of 475 nm against a standard curve of known concentrations of synthetic melanin (Sigma). Melanin content was expressed as μg/10⁶ cells. All experiments were performed in triplicate.

Statistical analysis

All experiments were performed in triplicate. Values are expressed as means ± SD in the results and calculations. Densitometric quantification of all blots was performed with β-actin as the endogenous control. For the calculation of the ratios of proteins, the densitometric values for each blot were standardized against a control of 1.0. Two-way analysis of variance (ANOVA) testing was performed for comparisons between two and among three or more groups. If a significant difference of P values < 0.05 was detected with the two-way ANOVA test, then a t test was applied for comparisons between groups.
Results

Nitric oxide assay

Unconcentrated culture medium (200 μl) was used (n = 5 in each group) to assay nitric oxide concentration. The concentration of nitric oxide was 8.819 ± 1.541 μM in the control group, 25.124 ± 1.233 μM in the UV group, and 6.683 ± 2.864 μM in the UV + L-NAME group. The results showed that the UV group can produce more NO than the other groups (P < 0.05). L-NAME can significantly reduce the concentration of NO in alpaca skin melanocytes under UV radiation. [22]

The expression of MITF

We tested the expression of MITF in each group using an indirect immunofluorescence microscopy method. The MITF-positive staining was found in all three groups, and the MITF-positive staining in the UV group was not significantly different from the other two groups (Fig. 1). We also tested the protein expression level of MITF and the mRNA expression level of MITF by western blotting and quantitative real-time PCR, respectively. The immunoblot showed MITF at 60 kDa. For the UV group, the MITF protein level was not substantially altered as compared with the control and the UV + L-NAME group (P > 0.05) (Fig. 2). MITF mRNA levels were also not substantially altered as compared with the control and the UV + L-NAME group (P > 0.05) (Fig. 3).

Expression of p-MITF

We investigated the molecular response potential, as indicated by the presence or absence of NO, by immunoblot analysis of p-MITF proteins (Fig. 2). The immunoblot showed p-MITF at 67 kDa (Fig. 1a). The p-MITF protein level of the UV group was significantly elevated compared to the other two groups (1.74-fold above the control group and 2.19-fold above the UV + L-NAME group (P < 0.05)). As a result, p-MITF protein substantially increased or decreased in accordance with increase or decrease in NO concentration.

Melanin content

The melanin content was 10.763 ± 3.014 μg/10^6 cells in the control group, 42.621 ± 5.193 μg/10^6 cells in the
UV group, and 21.683 ± 4.769 μg/10⁶ cells in the UV + L-NAME group. The results showed that the UV group can produce more melanin when compared with the other groups (P < 0.05). NO can significantly elevate the melanin content in alpaca skin melanocytes under UV radiation (Fig. 4).

**Discussion**

Melanin is synthesized by specific tyrosinase and tyrosinase-related enzymes that are expressed in melanocytes. Our previous research results demonstrated that TYR and DCT were substantially elevated or decreased following an increase or decrease in NO concentration [22]. Sasaki and Roméro-Graillet obtained similar results [13, 24]. Results in the current study show that NO can significantly elevate the melanin content in alpaca skin melanocytes under UV radiation (Fig. 4).

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When MITF is phosphorylated, it in turn regulates the transcription of genes coding MRPs through interactions with M- and E-boxes present in the promoter regions of TYR, TYRP-1, and DCT [6, 25–29]. Significant changes were not observed in MITF protein in the presence or absence of NO in our models, so the presence of NO alone cannot up-regulate the MITF in melanocytes. However, how it can up-regulate TYR and DCT to enhance melanogenesis is still unresolved, so p-MITF was examined. The results showed that p-MITF was significantly elevated in the UV group compared with other groups (P < 0.05). We gather from this result that nitric oxide was produced by UV-induced melanocytes, which then stimulated phosphorylation of MITF. This led to increased TYR, TYRP-1 and DCT expression, and ultimately enhanced the production of melanin.

In conclusion, this study provides exciting new evidence for the theory that NO can enhance melanogenesis in alpaca skin melanocytes, not by elevating the MITF, but by activating the MITF phosphorylation.
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References