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Nitric oxide enhances the sensitivity of alpaca melanocytes to respond to α-melanocyte-stimulating hormone by up-regulating melanocortin-1 receptor

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ABSTRACT

Nitric oxide (NO) and α-melanocyte-stimulating hormone (α-MSH) have been correlated with the synthesis of melanin. The NO-dependent signaling of cellular response to activate the hypothalamopituitary proopiomelanocortin system, thereby enhances the hypophysial secretion of α-MSH to stimulate α-MSH-receptor responsive cells. In this study we investigated whether an NO-induced pathway can enhance the ability of the melanocyte to respond to α-MSH on melanogenesis in alpaca skin melanocytes *in vitro*. It is important for us to know how to enhance the coat color of alpaca. We set up three groups for experiments using the third passage number of alpaca melanocytes: the control cultures were allowed a total of 5 days growth; the UV group cultures like the control group but the melanocytes were then irradiated everyday (once) with 312 mJ/cm^2 of UVB; the UV + L-NAME group is the same as group UV but has the addition of 300 μM L-NAME (every 6 h). To determine the inhibited effect of NO produce, NO produces were measured. To determine the effect of the NO to the key protein and gene of α-MSH pathway on melanogenesis, the key gene and protein of the α-MSH pathway were measured by quantitative real-time PCR and Western immunoblotting. The results provide exciting new evidence that NO can enhance α-MSH pathway in alpaca skin melanocytes by elevated MC1R. And we suggest that the NO pathway may more rapidly cause the synthesis of melanin in alpaca skin under UV, which at that time elevates the expression of MC1R and stimulates the keratinocytes to secrete α-MSH to enhance the α-MSH pathway on melanogenesis. This process will be of considerable interest in future studies.

1. 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. These melanogenic factors include nitric oxide (NO) [1], adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH) [2,3], endothelin-1 (ET-1) [4,5], prostaglandins [6], thymidine dinucleotide [7], and histamine [8]. Some of them are reported to up-regulate tyrosinase gene expression through different pathways.

NO is considered to play an important role in UV-induced hyperpigmentation [1,9]. It 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) [10–12]. 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 [1]. It may also be involved in the induction of the tyrosinase mRNA message, which is induced within 2 h of an application *in vitro* of an NO donor [9]. Other NO-activated pathways, however, might also be important. In the presence of oxygen, NO reacts with the melanin related metabolites 5,6-dihydroxyindole and its 2-carboxylic acid (DHICA) resulting in the deposition of melanin-like pigments [13]. Evidence from other cell systems suggests that NO can reduce inositol triphosphate (IP3) synthesis [14] and reduce the release of Ca^{2+} via modulation of the IP3 receptor [15,16]. The enhancement of tyrosinase gene expression via the cyclic guanosine 3′,5′-monophosphate (cGMP) pathway may be a primary mechanism for NO-induced melanogenesis [9].

α-MSH is reported to regulate melanogenesis via the cyclic adenosine 3′,5′-monophosphate (cAMP) pathway. Downstream melanocortin-1 receptor (MC1R) signaling events mediated by agonist treatment with the α-MSH hormonal peptide include cAMP accumulation [17], activation of the pigmentary transcription factor microphthalmia-associated transcription factor (MITF)

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[18], increased levels of the major pigmentation protein tyrosinase (TYR), dopachrome tautomerase (DCT) and tyrosinase related protein-1 (TYRP-1) [19]; as well as an increase in dendricity [20], pigment production [19,21,22], and cell growth [19,22,23].

It is not known whether an NO-induced pathway can affect an α-MSH induced pathway. Recently, Keiichi Hiramoto used UVB to stimulate the eyes and ears of mice, and the results show that the NO-dependent signaling of cellular response to activate the hypothalamopituitary proopiomelanocortin system, thereby enhancing the hypophysial secretion of α-MSH to stimulate α-MSH-receptor responsive cells [24]. But it is not known whether an NO-induced pathway can enhance the ability of melanocytes to respond to α-MSH on melanogenesis *in vitro*. This is important for us to know in order to enhance the coat color of alpaca.

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 N^G-nitro-L-arginine methylester hydrochloride, L-NAME, a specific NOS inhibitor) on proopiomelanocortin (POMC, α-MSH precursor), MC1R, and melanogenesis-related genes. This can help us to understand the molecular and signaling mechanisms.

2. Materials and methods

2.1. 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 [25]. Melanocytes in the culture were maintained in a 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.

2.2. 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 cultures like the control but the melanocytes were then irradiated every day (once) with 312 mJ/cm^2 of UVB from an SE lamp (FL 20 SE40 lamp, Toshiba, Tokyo, Japan); the UV + L-NAME group is the same to group UV but addition of 300 μM L-NAME (every 6 h). After 5 days we harvest the samples. Using the three groups, two experimental protocols were designed. Protocol A for identifying the inhibitory effect of L-NAME on NO, we assessed by measuring the concentration of nitric oxide in the culture medium. Protocol B assessed the effect of NO on an α-MSH induced melanin synthesis pathway. Quantitative real-time PCR was used to determine the mRNA of POMC, MC1R, TYR, TYRP-1, and DCT. Western blot analysis was used to determine the protein of MC1R, TYR, TYRP-1, and DCT.

2.3. Nitric oxide assay

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

2.4. RNA preparation and quantitative real-time PCR

To confirm the mRNA expression levels of POMC, MC1R, TYR, TYRP-1, and DCT, we conducted quantitative real-time PCR. Total RNA was obtained from melanocytes using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions (n = 3 in each group). The reverse transcriptase reaction was performed using 1 μg 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 the SYBR® Premix Ex Taq™ II (Takara, Dalian, China) and the Agilent MX3005P QPCR System according to the manufacturer's instructions. We designed POMC, MC1R, TYR, TYRP-1, and TYRP-2 and 18s RNA (endogenous control gene) primer and they are shown in Table 1. The conditions for real-time PCR were preheating at 95 °C for 10 s, followed by 40 cycles of shuttle heating at 95 °C for 15 s at 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 levels. All samples were run in triplicate. Quantitation of gene expression by real time-PCR was evaluated using the Comparative CT Method according 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.
(3) $2^{-\Delta\Delta CT}$, which yields the relative mRNA units representing the fold induction over the control.

2.5. Cell extraction and Western blotting

After treatments, the 6 cm dishes were placed on ice, rinsed with ice-cold PBS, 400 μl cell disruption buffer (RIPA Lysis Buffer, Beyotime, Shanghai, China), and 1 mmol/l PMSF added and sonicated for 10 s on ice. Cell lysates were centrifuged at 14,000g 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-MC1R (N19, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-TYR (H109, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-TRP1 (H90, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-DCT (TRP2 H150, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (1:1000, CWBIO, Beijing, China). Dilutions were all in fresh 5% skim milk blocking buffer and incubations were overnight at 4 °C. After washing in TBS, 0.1% with Tween-20, the appropriate horse radish peroxidase-conjugated secondary antibody (Zymed Laboratories, Inc.,

Table 1
Sequence of primer and PCR amplification of target gene.

Target gene	Sequence premier (5′ → 3′)	Products/bp
MC1R	F: CTATGCACTGCGCTACCAC R: ACATATAGCACCGCCATGA	185
TYR	F: CTGGACCTCAGTTCCCCTTC R: ACTTACAGTTTCCGCAGTTGA	121
POMC	F: CCCTACAGGATGGAGCACTTC R: GATGGCGTTTTTGAACAGCGT	117
TYRP-1	F: GCCTTCTTTCTCCCTTC R: CAGACCACTCGCCATT	100
DCT	F: TGCTTTGCCCTACTGGAAC R: TCAGAGTCGATCGTCTG	150
18S rRNA	F: GAAGGGCACCACCAGGAGT R: CAGACAAATCACTCCACCAA	158

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.

2.6. 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, respectively. If a significant difference of p values <0.05 was detected with the two-way ANOVA test, a t-test was then applied for comparisons between groups.

3. Results

3.1. Nitric oxide assay

About 200 μl of supernatant culture medium was obtained ($n = 5$ in each group) to assay nitric oxide concentration. The concentration of nitric oxide was 8.819 ± 1.541 μM in control, 25.124 ± 1.233 μM in the UV group, and 6.683 ± 2.864 μM in the UV + L-NAME group. The results show 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.

3.2. Determination of the key protein associated with pigmentation

We investigated the potential for molecular responses following the presence or absence of NO under UV condition by immunoblot analysis of the major proteins in α-MSH pathway associated with pigmentation (Fig. 2). In the immunoblot shown MC1R 34 kDa, TYR 70–90 kDa, TYRP-1 85 kDa, DCT 59 kDa, and β-actin 43 kDa (Fig. 1A). For the UV group MC1R protein levels were significantly elevated 2.9-fold above the control and 7-fold above the UV + L-NAME group; TYR protein levels were increased 1.2-fold above the control and 2.6-fold above the UV + L-NAME group; TYRP-1 protein levels were increased 3.9-fold above the control and 0.3-fold above the UV + L-NAME group; DCT protein levels were increased 1.2-fold above the control and 1-fold above the UV + L-NAME group; when the concentration of NO was elevated or decreased, the MC1R, TYR, and DCT protein were substantially elevated or decreased. Though TYRP-1 was elevated under UV condition as compared to the control, but the UV group was increased 0.3-fold above the UV + L-NAME group. NO may not be the chief regulator on the protein expression of TYRP-1 under UV condition.

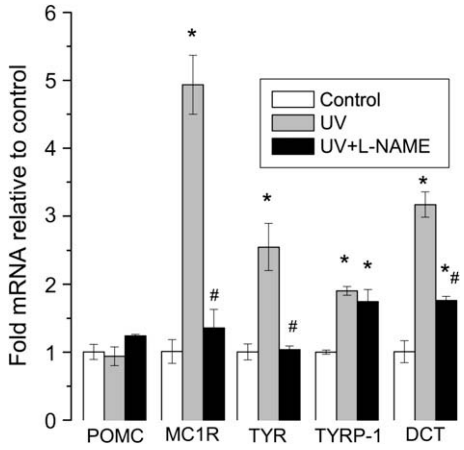

Fig. 2. As the key genes on melanogenesis, MC1R, TYR, TYRP-1, and DCT were assessed by quantitative real-time PCR. Control, normal cultured group; UV, cultured group under UV condition; UV + L-NAME, L-NAME-treated group under UV condition. $^{*}p < 0.05$ vs. control, $\#p < 0.05$ vs. the UV group.

3.3. Determination of the key mRNA associated with pigmentation

We also investigated whether the mRNA level had a similar change as the protein. For the UV group MC1R mRNA levels were increased 4-fold above the control and 3.8-fold above the UV + L-NAME group; TYR mRNA levels were increased 1.5-fold above the control or the UV + L-NAME group; DCT mRNA levels were increased 2.4-fold above the control and 0.8-fold above the UV + L-NAME group; TYRP-1 mRNA levels were increased 1.1-fold above control, but had no substantial change compared with the UV + L-NAME group. Significant elevation was shown on MC1R, TYR, and DCT mRNA levels. TYRP-1 levels were elevated under

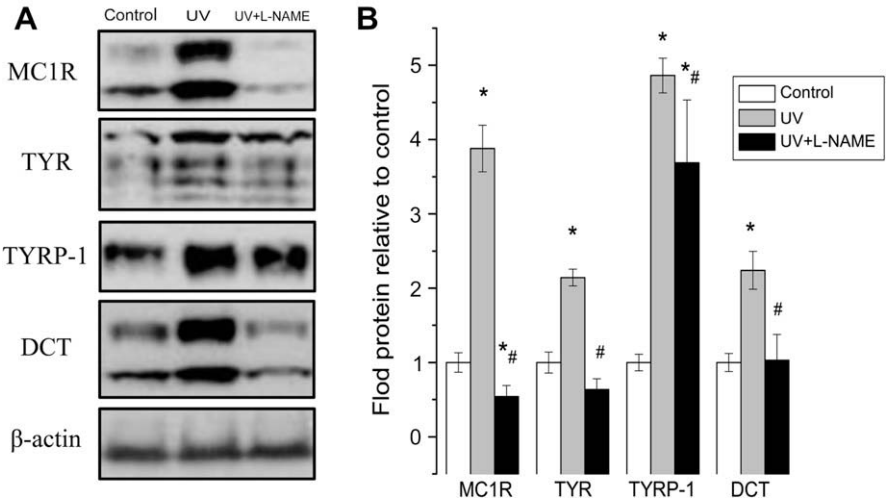

Fig. 1. As the key proteins on melanogenesis, MC1R, TYR, TYRP-1, and DCT were assessed by Western blot. Control, normal cultured group; UV, cultured group under UV condition; UV + L-NAME, L-NAME-treated group under UV condition. $^{*}p < 0.05$ vs. control, $\#p < 0.05$ vs. the UV group.

UV condition, but the presence or absence of NO cannot affect TYRP-1 mRNA levels. We also determined the POMC mRNA, which is α-MSH precursor, and it did not substantially changed with UV and NO presence or absence (Fig. 2).

4. Discussion

Melanin is synthesized by specific tyrosinase and tyrosinase-related enzymes, which are expressed in melanocytes. The results of our research demonstrate that the TYR and DCT were substantially elevated or decreased following the increase or decrease of the concentration of NO. Sasaki and Roméro-Graillet also got similar results [9,26]. We suggest the TYR and DCT expression of alpaca skin melanocyte cells relies on NO, which is cultured *in vitro* under UV condition (Figs. 1 and 2). These observations indicate that alpaca melanocyte cell culturing *in vitro* under UV condition can be used as a model system in pigmentary studies to investigate the basic potential mechanisms, by which NO affect other melanogenesis pathways.

L-NAME is a perfect NOS inhibitor. Lawrence and Brain reported that the intradermal injection of the NOS inhibitor, L-NAME, significantly reduced flow rates in rat skin [27]. *In vivo* experiments on guinea pigs have shown that the topical application of L-NAME inhibits UV-induced melanogenesis, reducing melanin content and the number of histochemically positive melanocytes [28]. In our study, L-NAME is good at inhibiting the NO production and melanogenesis.

α-Melanocyte-stimulating hormone (α-MSH) is a peptide derived from proopiomelanocortin (POMC) [29]. We detected POMC mRNA levels in the control, UV, and UV + L-NAME groups. The results show that POMC is not obviously changed among the three groups (Fig. 2). NO and UV can not affect the expression of POMC in these models, and α-MSH also can not make the difference of downstream genes among the three groups. In this study, we used alpaca melanocyte cell and L-NAME cultured *in vitro* under UV condition to create models to research whether NO can affect the melanogenesis pathway of α-MSH.

MC1R is major receptor for α-MSH in the melanogenesis pathway. We researched the effect of NO on MC1R expressing. Within cells of the skin, MC1R is located specifically on melanocyte membranes [30–33] where it functions in switching the type of melanin produced from the red/yellow pheomelanins, to the black/brown eumelanins [34,35]. Eumelanin production confers greater protection to skin cells from UV radiation through means including increased photo-absorption accompanied with lower phototoxic biproduct formation [36,37]. For this reason MC1R plays a central role in determining pigmentation phenotype, sun sensitivity, and tanning ability [38]. Upregulation of MC1R gene expression by α-MSH in cultured murine melanocytes have been reported previously [39] and may therefore enhance the sensitivity of cells to melanogenic stimulating. In our results, NO can remarkably elevate the expression of MC1R, and enhance sensitivity of cells to melanogenic stimulating; therefore, the α-MSH pathway will be enhanced. The results of Keiichi Hiramoto clearly indicate that a signal evoked by ultraviolet B irradiation of the eye is transmitted in a nitric oxide-dependent manner through the ciliary ganglia involving the first branch of the trigeminal nerve to the hypothalamopituitary proopiomelanocortin system, resulting in upregulation of a-melanocyte-stimulating hormone secretion and consequent stimulation of melanocytes in the skin [24]. It is an interesting result, but it does not consider whether NO can affect the ability of melanocytes to response to α-MSH. According to the result of Keiichi Hiramoto and our study, we suggest that the NO pathway elevates the expression of MC1R and stimulates the secretion of α-MSH to enhance the α-MSH pathway on melanogenesis.

In conclusion, this study provides exciting new evidence for that NO can enhance the α-MSH pathway in alpaca skin melanocytes by elevated MC1R. And we suggest that the NO pathway may more rapidly cause the synthesis of melanin in alpaca skin, which at that time elevates the expression of MC1R and stimulates the keratinocytes to secrete α-MSH to enhance the α-MSH pathway on melanogenesis. This process will be of considerable interest in future studies.

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