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Endothelin-1 enhances the melanogenesis via MITF-GPNMB pathway

Ping Zhang[1,2,#], *Wei Liu*[1,2,#,*], *Xiaoying Yuan*[2], *Dongguang Li*[2], *Weijie Gu*[2] *& Tianwen Gao*[1]

[1]Department of Dermatology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, [2]Department of Dermatology, The Air Force General Hospital of PLA, Beijing 100142, China

Endothelin-1 (ET-1) plays an indispensable role in epidermal pigmentation in hyperpigmentary disorders due to a central role in melanogenesis. Nevertheless, precise mechanism involved in ET-1-induced hyperpigmentation is still undefined. Glycoprotein (transmembrane) non-metastatic melanoma protein b (GPNMB) is a key element in melanosome formation. Therefore, we speculated that GPNMB was correlated with ET-1-induced pigmentation. After culturing with ET-1, melanin synthesis was significantly up-regulated, accompanying with increased expression of GPNMB and microphthalmia- associated transcription factor (MITF). Total number of melanosomes and melanin synthesis were sharply reduced via GPNMB-siRNA transfection, indicating ET-1-induced pigmentation by GPNMB-dependent manner. Furthermore, MITF-siRNA transfection strikingly inhibited GPNMB expression and the melanogenesis, and this suppression failed to be alleviated by ET-1 stimulation. All of these results demonstrated that ET-1 can trigger melanogenesis via the MITF-regulated GPNMB pathway. Taken together, these findings will provide a new explanation of how ET-1 induces hyperpigmentation, and possibly supply a new strategy for cosmetic studies. [BMB Reports 2013; 46(7): 364-369]

INTRODUCTION

Skin pigment is known as a critical element in the protection of organism. An excessive or reduced production of melanin in skin accounts for pigment-related diseases, including of melanoma, vitiligo, seborrheic keratosis (SK), and so on (1-3). In recent years, more attention has been paid to this due to the relationship between increased pigmentation and cosmetic study (4).

*Corresponding author. +86-10-66928074; Fax: +86-10-68485082; E-mail: weiliuair@gmail.com
[#]Both of these two authors contributed equally to this work.

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Pigment production is induced by light. It is commonly that UV-induced hyperpigmentation is composed of three major steps: the first step is the proliferation of melanocytes (5), followed by increased melanogenesis, and ultimately, the transfer of melanosomes from melanocytes to keratinocytes (6, 7). In recent years, the relationships between endothelins and pigment diseases have drawn considerable attention due to the indispensable role of endothelins in epidermal pigmentation in several hyperpigmentary disorders (8, 9). As intrinsic mediators for human melanocytes, endothelins play vital roles in UVB-induced pigmentation (8). Among these endothelin peptides, endothelin (ET)-1 is considered to be an important member. ET-1 was firstly isolated from vascular endothelial cells (10), and can induce mitogenesis and melanogenesis in primary human melanocytes (11). The increased secretion of ET-1 is intrinsically considered to be involved in the hyperpigmentation mechanism of SK (9, 12). However, the precise mechanisms involved in this process are lacking. Therefore, to clarify the mechanism of endothelins involved in hyperpigmentation will be useful for the treatment of pigment-related diseases and study of cosmetics.

It is well known that synthesis and transfer of melanin are pivotal in the study of pigmentary skin diseases and cosmetic problems. Many melanosome-related proteins should be considered due to the crucial roles in this process. It has been reported that melia toosendan dramatically attenuates ET-1-stimulated pigmentation by inhibiting the phosphorylation of Protein Kinase C (PKC) downstream targets, including microphthalmia transcription factor (MITF) (13). MITF is a key transcription factor involved in the differentiation, growth and survival of melanocytes, can regulate more than 25 pigmental genes, including tyrosinase (Tyr), Pmel17, glycoprotein non-metastatic melanoma protein B (GPNMB) and so on (14-16). GPNMB was initially identified from poorly metastatic melanoma cells (17). It exhibits a high similarity with a well-known melanosomal structural protein, Pmel17 (18). GPNMB contains several domains related with its functions in melanocytes (18). It has been confirmed that the arginine-glycine-aspartate (RGD) motif of GPNMB can bind to integrins to regulate the adhesion of melanocytes with keratinocytes, indicating that it is involved in the transfer of melanin (19). As an important

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structural protein of melanosomes, GPNMB has proven to be present in all stages (I-IV) of melanosomes, and is especially enriched in mature stages (18, 20). In our previous works, GPNMB deletion in melanocytes sharply attenuated melanosome formation, indicating a critical role in melanosome synthesis (21). Simultaneously, ET-1 can elevate the expression levels of Try and Trp1, contributing to UVB-induced pigmentation (8). Whether there is a correlation between ET-1 and another melanosome structural protein-GPNMB in hyperpigmentation has not been reported. So we hypothesized that endothelin-induced hyperpigmentation is related with melanosome-specific protein GPNMB.

To address this hypothesis, human melanocytes were stimulated with ET-1 *in vitro*. The relationships between ET-1 and GPNMB and MITF were analyzed to clarify the mechanism about ET-1-induced hyperpigmentation.

RESULTS AND DISCUSSION

ET-1 enhanced melanogenesis accompanying with the up-regulation of GPNMB expression

To analyze the relationship between ET-1 and melanogenesis, human melanocytes were stimulated with various concentrations of ET-1 for 1, 3, or 5 days. A clear time- and dose-dependent correlation between ET-1 concentration and melanin formation was confirmed. As shown in Fig. 1A, treatment with high doses of ET-1 enhanced the melanin formation, and a strikingly higher concentration of melanin was detected when treated with 50 nM ET-1. In addition, similar patterns of time-dependent increases of melanogenesis were also observed in ET-1 cultured cells, and a striking increase of melanin synthesis was detected from day 5.

Whether ET-1-induced melanin formation was related with GPNMB production. To address this problem, the mRNA and protein levels of GPNMB were analyzed by real-time PCR and western blotting. As shown in Fig. 1B, the transcription of GPNMB mRNA was sharply up-regulated, accompanying with increased ET-1 concentrations. Consistent with the above observation, rapid increase of GPNMB protein levels were corroborated via ET-1 stimulation (Fig. 1C), but this lagged behind the expression of GPNMB mRNA. Moreover, the similar time-dependent expression patterns of GPNMB mRNA and protein levels were also observed. Simultaneously, the high expression levels of GPNMB induced by ET-1 were also demonstrated by immunofluorescence microscopy, and large positive signals were detected compared with the control group (Fig. 1D). Together, these data demonstrated that ET-1-induced melanin synthesis was accompanied with a large amount of GPNMB mRNA and protein expression.

GPNMB knockdown reduced ET-1-induced melanogenesis in melanocytes

Whether ET-1-induced melanogenesis were associated with GPNMB expression. To clarify this question, specific GPNMB-siRNA and scrambled-siRNA were transfected into melanocytes, and real-time PCR and western blotting were used to evaluate the silencing effect of GPNMB. Transfected with GPNMB-siRNA caused the mRNA and protein levels of GPNMB to decrease to about 19.2% (Fig. 2A) and 24.5% (Fig. 2B), respectively, indicating an obvious decline of GPNMB expression compared to the non-treatment control. Accompanied with these changes, melanin content was significantly reduced

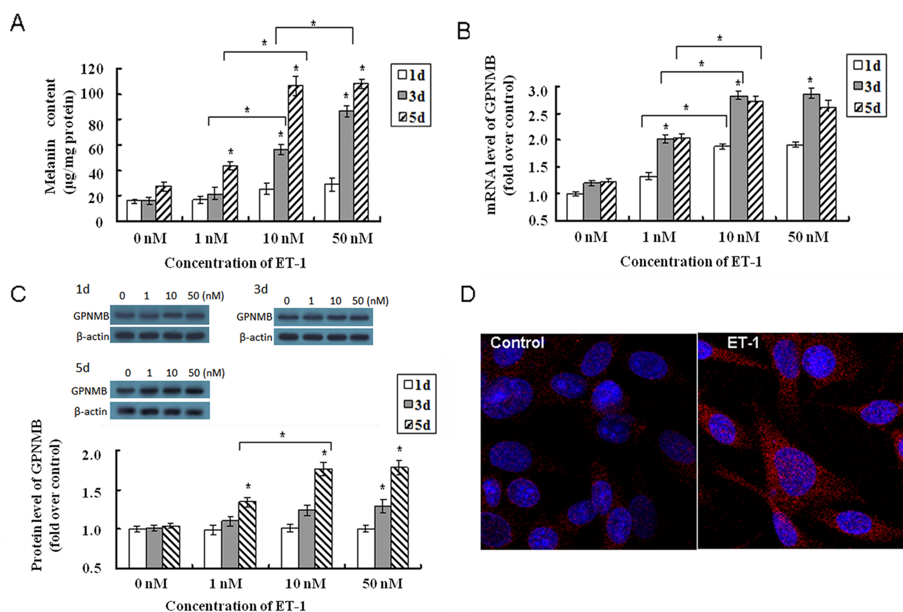

Fig. 1. ET-1 induced adequate melanin synthesis and GPNMB expression in human melanocytes. (A) Melanin content in melanocytes after being treated with ET-1 for various doses and times. *P < 0.05. (B) After incubation with ET-1, the mRNA levels of GPNMB were detected by real-time PCR. β-actin was used as a reference. (C) The corresponding GPMB protein levels were analyzed via western blotting. (D) Immunofluorescence was performed with phycoerythrin (PE)-conjugated (red) antibody to detect GPNMB expression. DAPI (blue) was introduced to determine the nuclear position for visualization purposes. Fluorescence was analyzed using a fluorescence microscope.

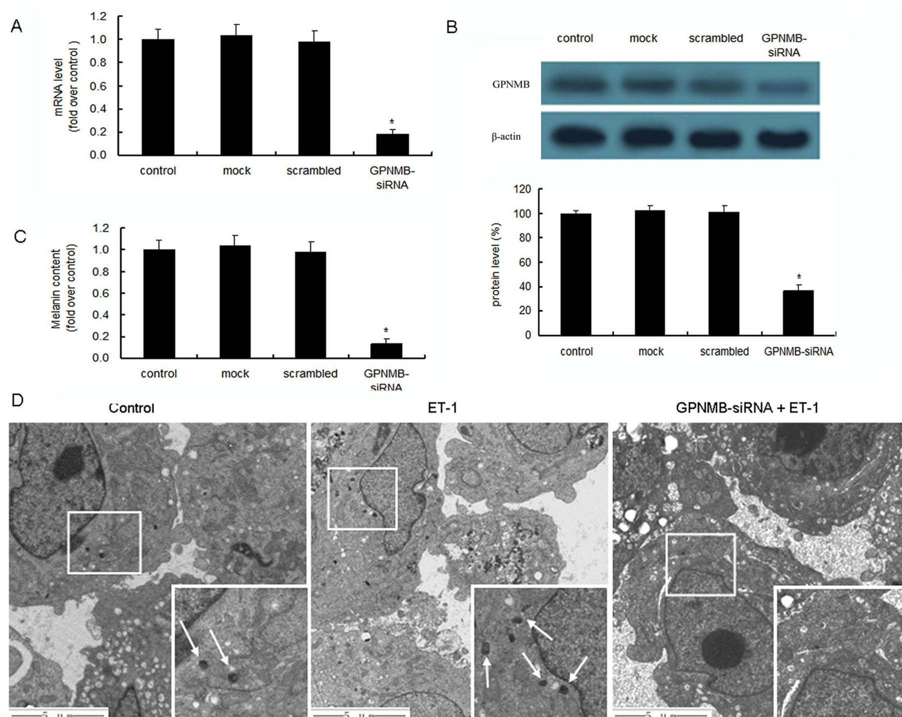

Fig. 2. GPNMB knockdown reduced ET-1 induced melanogenesis. The GPNMB siRNA and scrambled siRNA were transfected into melanocytes. Following this treatment, cells were cultured in medium containg 50 nM ET-1 for 5 days. The silencing effect of GPNMB mRNA levels and protein levels was detected by real-time PCR (A) and western blotting (B), respectively. Data were normalized based on the β-actin levels. To analyze the effect of GPNMB-siRNA on melanogenesis, the melanin content was detected (C). Furthermore, the melanosome formation was analyzed by Transmission electron microscopy (TEM) (D). *P < 0.05 by Student's *t* test when compared with the control group.

due to the silence of GPNMB expression (Fig. 2C). Transmission electron microscopy (TEM) analysis showed that ET-1 stimulation could evidently promote the formation of melanosomes compared with un-treated melanocytes (Fig. 2D). After the knockdown of GPNMB, numerous formations of melanosomes were inhibited in the vast majority of melanocytes, and even when stimulated with ET-1, this suppression failed to be alleviated. Taken together, all of these results confirm the hypothesis that ET-1 regulates melanogenesis mainly in a GPNMB-dependent manner. However, GPNMB silencing almost abrogated melanosome formation in our previous work, indicating a crucial role in the formation of melanosomes (21). Whether GPNMB knockdown abolishes melanin formation by a direct effect on melanosome formation, or ET-1 activated signal pathway remains to be determined. Further studies will be needed to clarify this question.

ET-1 induced melanogenesis via an MITF-GPNMB-dependent manner

Endothelin is known to modulate MITF phosphorylation in an endothelin receptor B-dependent manner (22). Furthermore, endothelin-induced MITF phosphorylation can also be activated by MAPK pathway (23). The activated signal transducer MITF stimulates target gene expression as the primary mechanism in melanogenesis (14, 24). In melanocytes, MITF is thought to be an essential transactivator, accomplishing the assembly of the melanosome and its decoration with melanin, and is thought to regulate more than 25 pigmentation genes,

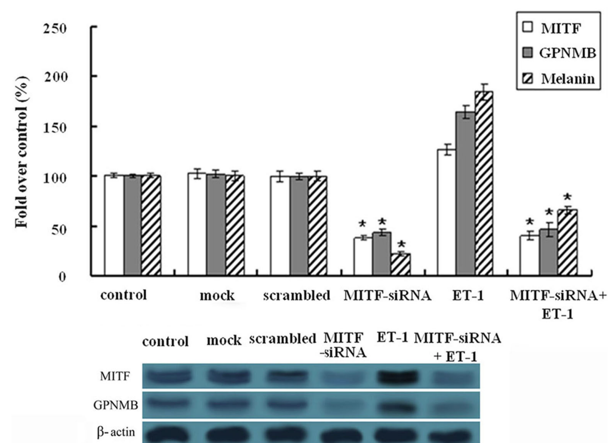

Fig. 3. ET-1 induced melanogenesis via a MITF-GPNMB dependent manner. After treated with MITF-siRNA or scrambled siRNA, the protein levels of MITF and GPNMB were analyzed by western blotting at 5 day of ET-1 stimulation. Furthermore, the influence on melanin formation was also detected. When cultured with ET-1, the corresponding GPNMB expression and melanin synthesis were determined. While, MITF-siRNA or both MITF-siRNA and ET-1stimulation had little difference on GPNMB expression and melanogenesis. *P < 0.05.

including tyrosinase (Tyr), Pmel17, GPNMB and so on (14). As one of the downstream effectors of MITF, GPNMB had been proved to be associated with ET-1-induced melanogenesis in

the present work, so it was speculated that GPNMB-induced ET-1 pathway was regulated in an MITF-dependent manner. As shown in Fig. 3, transfection with MITF-siRNA sharply attenuated the expression of GPNMB and MITF. This study also found that numerous expressions of MITF and GPNMB were detected in ET-1 cultured cells compared with control cells, accompanied with abundant melanogenesis. Furthermore, MITF siRNA-transfection strikingly suppressed the melanogenesis, and this suppression failed to be alleviated by ET-1 stimulation. All of these results indicated that ET-1-induced GPNMB-dependent melanogenesis is regulated by MITF.

In conclusion, this study confirmed that ET-1 induced melanogenesis via the regulation of MITF-GPNMB. Therefore, it is easy to imagine that control of the MITF-GPNMB pathway is a key point in the treatment of pigmented dermal diseases, including of melanoma. Simultaneously, these findings may provide a new strategy for cosmetic study. However, the precise mechanism of interaction between ET-1 and MITF-GPNMB in melanogenesis is still unknown. Further attentions should be focused on this aspect.

MATERIALS AND METHODS

Cell culture

Primary human melanocytes were isolated from foreskin skin. All human material was obtained and processed according to the recommendations of the Air Force General Hospital of Chinese PLA. The isolated human melanocytes were cultured in melanocyte growth medium, consisting of 1 ng/ml recombinant basic fibroblast growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate, 50 μg/ml streptomycin, and 0.2% (v/v) bovine pituitary extract at 37°C with 5% CO_2, as previously described (25). During this process, ET-1 was added to a final concentration of 0, 1, 10 or 50 nM for 1, 3, or 5 days. Melanocytes from the third to fifth passage were used in this experiment.

Silencing of GPNMB and MITF expression with small interference RNA (siRNA)

To specifically silence the expression of GPNMB and MITF, four pairs of GPNMB-siRNA (21) and five pairs of specific MITF-siRNA nucleotide sequences were designed as previously described (26). A scrambled siRNA sequence, as described previously, was also used (27). All of the sequences were synthesized by Genetimes Technology (Shanghai, China). Human melanocytes (6×10^5/ml) were transfected with 2 μg/ml GPNMB siRNA, MITF-siRNA or Scrambled II siRNA using the GeneSilencer® siRNA transfection reagent (GeneTherapy System, San Diego, CA), according to the manufacturer's instructions.

Melanin measurements

For melanin content determination, the harvested cells were lysed with NE-PERTM protein extraction reagent (Pierce), and were then dissolved in 1 M NaOH. The protein concentrations were determined by the BCA assay (Pierce, Rockford, IL). Total melanin was measured by a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer, London, UK) at 405 nm. Synthetic melanin (Sigma, St. Louis, MO, USA) was used as a standard. Three duplicates were used for analysis. The concentration of melanin was shown in terms of μg melanin per mg protein.

Real-time PCR

Total RNA was isolated from human melanocytes with Tripure Isolation Reagent (Roche Diagnostics, Mannheim, Germany). The obtained RNA (~4 μg) was reverse transcribed to synthesize first strand cDNA with the Oligo (dT) $_{18}$ primer using the cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Then, 2 μl of cDNA was used as a template, and the amplified primers of GPNMB and β-actin were used as previously described (21). For normalization, β-actin mRNA was used. SYBR Green I was used as the fluorochrome in real-time PCR amplification. The reaction conditions followed the instructions provided by the manufacturers of the SYBR Premix Ex Taq™ II Kit (Takara Bio Inc., Otsu, Japan).

Western blotting

To clarify the expression of GPNMB and MITF in human melanocytes stimulated with ET-1, western blotting was used as described (21). The collected protein concentrations were determined by the BCA assay (Pierce) and the proteins were electrophoresed on a 12% polyacrylamide gel, before being transferred onto a polyvinylidene difluoride (PVDF) membrane (Pharmacia, Piscataway, NJ). After blocking, the target proteins were probed with rabbit anti-GPNMB antibody or mouse anti-MITF antibody (C5) (1 : 1,000) (Abcam, Cambridge, UK) at 4°C. A secondary goat anti-rabbit IgG HRP-conjugated antibody (Abcam, Cambridge, MA) and goat anti-mouse IgG HRP-conjugated antibody (1 : 3,000) (Dako Cytomation, Glostrup, Denmark) were used. The bound antibodies were visualized by using the LumiGLo reagent (Pierce) and the levels of each protein relative to that of β-actin were analyzed.

Immunofluorescence microscopy

The immunofluorescence assay was performed as described previously (21). Briefly, after 5 day of stimulation with ET-1, cells were washed twice with PBS and fixed with methanol at −20°C for 15 min. Coverslips were incubated in blocking buffer containing the appropriate primary Ab (rabbit anti-GPNMB polyclonal Ab) at 4°C, and then cultured with phycoerythrin (PE)-conjugated goat anti-rabbit IgG. Following this, cells were incubated with 1 μg/ml fluorescent dye DAPI (Sigma-Aldrich, Saint Louis, MO) and PBS for 30 min to evaluate the nuclear position. Fluorescence was observed with an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) and analyzed with Olympus FV1000 software FV10-ASW version 2.1b.

Transmission electron microscopy (TEM)

The TEM analysis was implemented as reported previously (21). The ultrathin sections were obtained using an RMC-MT6000XL ultra-microtome and were stained with uranyl acetate and lead citrate. Samples were then examined under an electron microscope (JEM-1200EX, JEOL, Japan) at an accelerating voltage of 75 kV.

Data analysis

Datas were analyzed using SPSS 11.0 for Windows. A typical image from at least three similar experiments was presented. Statistical analysis was carried out using *t*-tests. $P < 0.05$ was considered statistically significant. All results are expressed as mean $\pm$ SEM.

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