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iNOS inhibits hair regeneration in obese diabetic (*ob/ob*) mice

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ABSTRACT

Previous studies have shown that androgenic alopecia is associated with metabolic syndrome and diabetes. However, the detailed mechanism whereby diabetes causes alopecia still remains unclear. We focused on the inflammatory response that is caused by diabetes or obesity, given that inflammation is a risk factor for hair loss. Inducible nitric oxide synthase (iNOS) is known to be upregulated under conditions of acute or chronic inflammation. To clarify the potential role of iNOS in diabetes-related alopecia, we generated obese diabetic iNOS-deficient (*ob/ob; iNOS-KO* mice). We observed that *ob/ob; iNOS-KO* mice were potentiated for the transition from telogen (rest phase) to anagen (growth phase) in the hair cycle compared with iNOS-proficient *ob/ob* mice. To determine the effect of nitric oxide (NO) on the hair cycle, we administered an iNOS inhibitor intraperitoneally (compound 1400 W, 10 mg/kg) or topically (10% aminoguanidine) in *ob/ob* mice. We observed that iNOS inhibitors promoted anagen transition in *ob/ob* mice. Next, we administered an NO donor (S-nitrosoglutathione, GSNO), to test whether NO has the telogen elongation effects. The NO donor was sufficient to induce telogen elongation in wild-type mice. Together, our data indicate that iNOS-derived NO plays a role in telogen elongation under the inflammatory conditions associated with diabetes in mice.

1. Introduction

There are many types of hair loss in patients, and even though the end result of hair loss is the same, the causes vary. Previous studies have suggested that there is an association between obesity and androgenic alopecia [1–3]. However, the detailed mechanism of hair loss in diabetes is not fully understood.

The inflammatory response is considered one of the causes of hair loss [4]. Inducible nitric oxide synthase (iNOS) is known to be upregulated under conditions related to acute inflammation, for instance, upon bacterial infection [5–7]. iNOS also is known to play a role in the pathophysiology of chronic inflammation-related diseases such as metabolic syndrome and diabetes mellitus [8–11]. Nitric oxide (NO) is a key messenger in the pathogenesis of inflammation [12]. While NO functions as a pleiotropic signaling molecule [13,14], excess amounts of NO lead to the formation of reactive nitrogen species, which in turn cause pathological (cytotoxic) effects. Previously, NO was thought to have a beneficial role in hair growth [15,16]. Specifically, NO has physiological (protective) effects such as vasodilation; therefore, it was believed that NO promotes hair growth by increasing blood flow around the hair root [15]. Furthermore, constitutive NOS enzymes (i.e., endothelial NOS and neuronal NOS) are considered inducers of anagen (a hair-growth phase) [17]. Alopecia areata, a prevalent inflammatory cause of hair loss, is considered a T cell-mediated autoimmune disease that targets hair follicles and interrupts hair regrowth [18,19]. Several studies have reported that NO affects the balance between Th1 and Th2 lymphocytes [20–22]. Therefore, NO donors have been considered for use as anti-alopecia areata treatments [16]. In contrast, cicatricial alopecia is characterized by different inflammatory conditions that result in hair loss [23]. Excess amounts of NO, produced in response to inflammation, may be a cause of hair loss; however, a specific role for iNOS in hair loss has not yet been defined.

Obese leptin-deficient (*ob/ob*) mice are considered a good

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genetic model for testing hair growth agents; notably, hair regeneration after depilation is delayed in these mice by more than 10 weeks compared with wild-type mice [24–26]. However, the mechanism(s) of hair growth inhibition in *ob/ob* mice are not fully understood. Therefore, we generated an *iNOS* knockout in *ob/ob* mice (creating *ob/ob*; *iNOS-KO* mice) to clarify the potential role of iNOS in diabetes-related alopecia. These mice exhibited potentiation of transition to the anagen stage of the hair cycle.

These previous reports and our observations prompted us to hypothesize that NO might elongate telogen (resting phase) in *ob/ob* mice. To test our hypothesis, we administered iNOS inhibitor intraperitoneally (1400 W, 10 mg/kg) or topically (10% aminoguanidine) to *ob/ob* mice. In separate experiments, we administered an NO donor [S-nitrosoglutathione, 20 mM, intradermal (ID)] to confirm whether NO has a telogen elongation effect in wild-type mice.

2. Materials and methods

2.1. Materials

We purchased 1400 W and S-nitrosoglutathione (GSNO) from Cayman Chemical (Paul, MN), aminoguanidine from Tokyo Chemical Industry (Tokyo, Japan), glycerol from Wako (Osaka, Japan), and other chemical reagents from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

This study was approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University (A2017-021C). The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Throughout the in-life phase, the mice were maintained at 25 °C, illuminated by 12:12-h light:dark cycles, and provided with free access to standard rodent chow and water.

Seven-week-old male C57BL/6J, C3H, and *ob/ob* (C57BL/6J back ground) mice were purchased from the Sankyo Laboratory (Tokyo, Japan) for use in this study. *iNOS* knockout mice in the C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME). To obtain *ob/ob; iNOS-KO* mice, crosses were performed as follows. First, we mated *ob* ± mice with *iNOS* knockout mice ($iNOS^{-/-}$). The resulting $ob^{+/-}$; *iNOS* ± mice were intercrossed to produce $ob^{+/-}$; $iNOS^{-/-}$ mice. We then intercrossed these $ob^{+/-}$; $iNOS^{-/-}$ mice to obtain $ob^{-/-}$; $iNOS^{-/-}$ mice.

To assess hair regeneration, the dorsal hair of each mouse was depilated (e.g., 2 cm × 5 cm patch) using depilatory cream (Klacie, Japan) at eight-week-old. Following hair removal (Day 0), the mice were regularly monitored and recorded by photography (EOS kiss X5; Canon, Tokyo, Japan) for 2–6 weeks. Regenerated hair could be seen as dark hair on pinkish-white shaved skin [27]. The hair regeneration area was quantified using the NIH ImageJ 1.50b software program (NTIS, Springfield, VA).

2.3. Administration of iNOS inhibitor or NO donor

For intraperitoneal (IP) dosing, mice were injected IP with 1400 W (10 mg/kg) or an equivalent volume of sterile saline on alternating days for six weeks. For topical dosing, 50 μL of 10% aminoguanidine (in 5% glycerol) or 5% glycerol alone were applied to depilated area topically on every weekday (5 days/week) for 4 weeks. For intradermal (ID) dosing, animals were administered in depilated area once by ID injection with 50 μL of 20 mM S-nitrosoglutathione (GSNO) in saline (right side) or sterile saline alone (left side).

2.4. Isolation of total RNA and quantitative real-time reverse transcription (RT) -PCR

At eight-week-old, the dorsal hair of each mouse was depilated under anesthesia. After depilation, the dorsal skin was harvested. Total RNA was isolated from skin samples with an RNeasy Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). RT-PCR analyses were performed with 10 ng of cDNA and TaqMan probes (Applied Biosystems) for *iNOS* (Mm00440502_m1) and *GAPDH* (Mm99999915_g1) using as TaKaRa PCR Thermal Cycler Dice (Takara Bio, Osaka, Japan) as previously described [28]. The gene expression of *iNOS* was evaluated through normalization to that of *GAPDH*, a constitutively expressed housekeeping gene encoding glyceraldehyde phosphate dehydrogenase.

2.5. Statistics

We evaluated the differences in hair regeneration rate and RNA expression by a two-tailed one-way ANOVA followed by Newman-Keuls method (for comparisons of >2 groups). The differences in hair regeneration rate using with iNOS inhibitor was evaluated by a two-tailed, non-paired Student's t-test (for comparisons of 2 groups). Statistical analysis was performed using JMP, version 13 (SAS Institute Inc. Cary, NC). A value of $p < 0.05$ was considered to be significant. Where applicable, values are expressed as the mean ± SEM.

3. Results

3.1. iNOS knockout ob/ob mice showed significantly faster hair regrowth than ob/ob mice

To determine whether iNOS deficiency potentiated the delayed hair cycle of *ob/ob* mice, we depilated the back hair of *leptin* and *iNOS* double knockout (*ob/ob*; *iNOS-KO*) mice and *ob/ob* mice with depilatory cream as previously described [29]. We observed subsequent hair regrowth weekly and measured the hair regeneration area at 4 weeks after depilation (Fig. 1A–B). There was no significant difference in hair regeneration area between C57BL/6J (wild-type, WT) and *iNOS-KO* mice. In contrast, *ob/ob; iNOS-KO* mice ($n = 8$) showed potentiated hair regrowth compared with *ob/ob* mice ($n = 4$) (Fig. 1B). *ob/ob*; *iNOS-KO* mice began to regenerate hair by 2 or 3 weeks after depilation, while *ob/ob* mice showed no change for over 6 weeks post-depilation. The results in *ob/ob* mice are consistent with previous observations by ourselves [29] and others [26].

3.2. Expression of iNOS mRNA was increased in the skin of ob/ob mice at telogen

To compare the mRNA expression of *iNOS*, we extracted total RNA from the skin, including epidermis, dermis, and fat, from WT and *ob/ob* mice in their telogen (8 weeks old). The mRNA expression of *iNOS* was significantly higher in the skin of *ob/ob* mice (Fig. 1C).

3.3. The effect of iNOS inhibitor treatment on hair regrowth by ob/ob mice

We tested whether treatment with an iNOS inhibitor also potentiates hair regeneration in *ob/ob* mice. We depilated the back hair of *ob/ob* mice with depilatory cream as described above. First, we administered 1400 W, a selective iNOS inhibitor, or saline IP on