Distribution of Constitutive Nitric Oxide Synthase Immunoreactivity and NADPH-Diaphorase Activity in Murine Telogen and Anagen Skin

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The freely diffusible radical nitric oxide is generated by nitric oxide synthase, and is a pleiotropic, bioregulatory molecule that regulates, e.g., the vascular tone, functions as a major neurotransmitter, and is involved in macrophage-mediated cytotoxicity and platelet aggregation. Constitutive nitric oxide synthase exhibits NADPH-diaphorase activity that can be demonstrated histochemically. To study whether this enzyme is present in mammalian skin during distinct phases of the murine hair cycle, we have examined cryosections of C 57 BL-6 mouse skin in telogen and depilation-induced anagen VI. Histochemical analysis of NADPH-diaphorase activity was complemented by immunohistology, using two specific rabbit antisera against constitutive neuronal nitric oxide synthase. Epidermis and the outer root sheath showed both immunoreactivity for the enzyme and NADPH-diaphorase activity, whereas dermal papilla and sebaceous glands displayed only strong NADPH-diaphorase activity, suggesting that this enzyme histochemical test measures additional enzymes besides nitric oxide synthase. Intrinsic nitric oxide synthase immunoreactivity was also detected by immunoblot in mouse skin homogenates, staining proteins of an apparent 160-kDa molecular weight. Compared to telogen skin, these immunoreactive proteins were quantitatively increased in anagen VI skin. Thus, our study suggests that defined epithelial compartments of normal murine skin are capable of synthesizing nitric oxide and that the molecule may be involved in skin physiology, growth, and remodeling. *Key words: Nitric oxide/NADPH-diaphorase/hair follicle. J Invest Dermatol 103:112–115, 1994*

Nitric oxide (NO), a diffusible free radical with a very short half life, is a pleiotropic, bioregulatory molecule with multiple physiologic functions [1]. It is, for example, the messenger of macrophage-dependent cell-mediated cytotoxicity, it functions as intercellular messenger in neural signaling, and it is an important mediator of blood vessel relaxation [2–4]. NO is synthesized from L-arginine by the NADPH-dependent nitric oxide synthase (NOS) [5]. The local activity of NOS determines therefore the NO concentration in any given tissue location. Signal transduction after NO stimulation involves soluble guanylyl cyclases as receptors that generate cGMP as a secondary messenger [6–8]. At least two distinct types of NOS are currently well known, a constitutive Ca^{++}/calmodulin-dependent enzyme (NOS-I) [9–11] and an inducible Ca^{++}-independent enzyme (NOS-II) whose synthesis is stimulated, e.g., by tumor necrosis factor (TNF)-alpha, interleukin (IL)-1, and endotoxin [3,12–14].

Although NOS activity has been found in almost every tissue investigated so far [1,15,16], surprisingly little is known on the role of NO and NOS in skin. *In vitro*, murine and human keratinocytes generate NO after stimulation with TNF-alpha and lipopolysaccharide [17], and a human keratinocyte line (HaCaT) displays NOS activity after stimulation with bradykinin.§ To study the presence of NOS-I activity and antigen in mammalian skin under physiologic circumstances and to detect whether any such activity may be developmentally regulated, we have examined cryosections of C 57 BL-6 mouse skin at two distinct hair cycle stages (telogen, depilation-induced anagen VI), using both enzyme histochemistry and immunohistology. Two different hair-cycle stages were investigated to assess whether NOS-I expression and activity is developmentally regulated in normal skin [18–23]. Because the cyclic activity of hair follicle growth (anagen), regression (catagen), and resting (telogen) is highly synchronized in C 57 BL-6 mice when anagen is induced mechanically by depilation [18–21], this model of depilation-induced murine hair cycle was chosen for the present studies. NOS activity was assayed by determining NADPH-diaphorase (NADPH-d) activity, which is based on the NADPH-dependent reduction of nitroblue tetrazolium (NBT) or tetranitroblue tetrazolium (TNBT) by NOS-I to the water-insoluble dye formazan [24,25]. NOS antigen was assayed by performing immunohistology with antisera raised against porcine [9] and rat cerebellar NOS-I [15]. In addition, immunoblots with NOS-I antisera [9] were performed on homogenates from telogen and anagen VI skin.

MATERIALS AND METHODS

Animal Model and Tissue Collection Mice with all of their back skin follicles in telogen, as recognized by the uniformly pink back skin color [22], were selected for study. These telogen follicles were induced to enter anagen by depilation of the hair shafts with a wax/rosin mixture, as previously

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; TNBT, tetranitroblue tetrazolium; TNBTF, tetranitroblue tetrazolium formazan.

§ Rosenbach T, Dippel E, Nolte B, Czarnetzki BM: HaCaT keratinocytes generate nitric oxide (abstr). *Arch Derm Res* 285:92, 1993.

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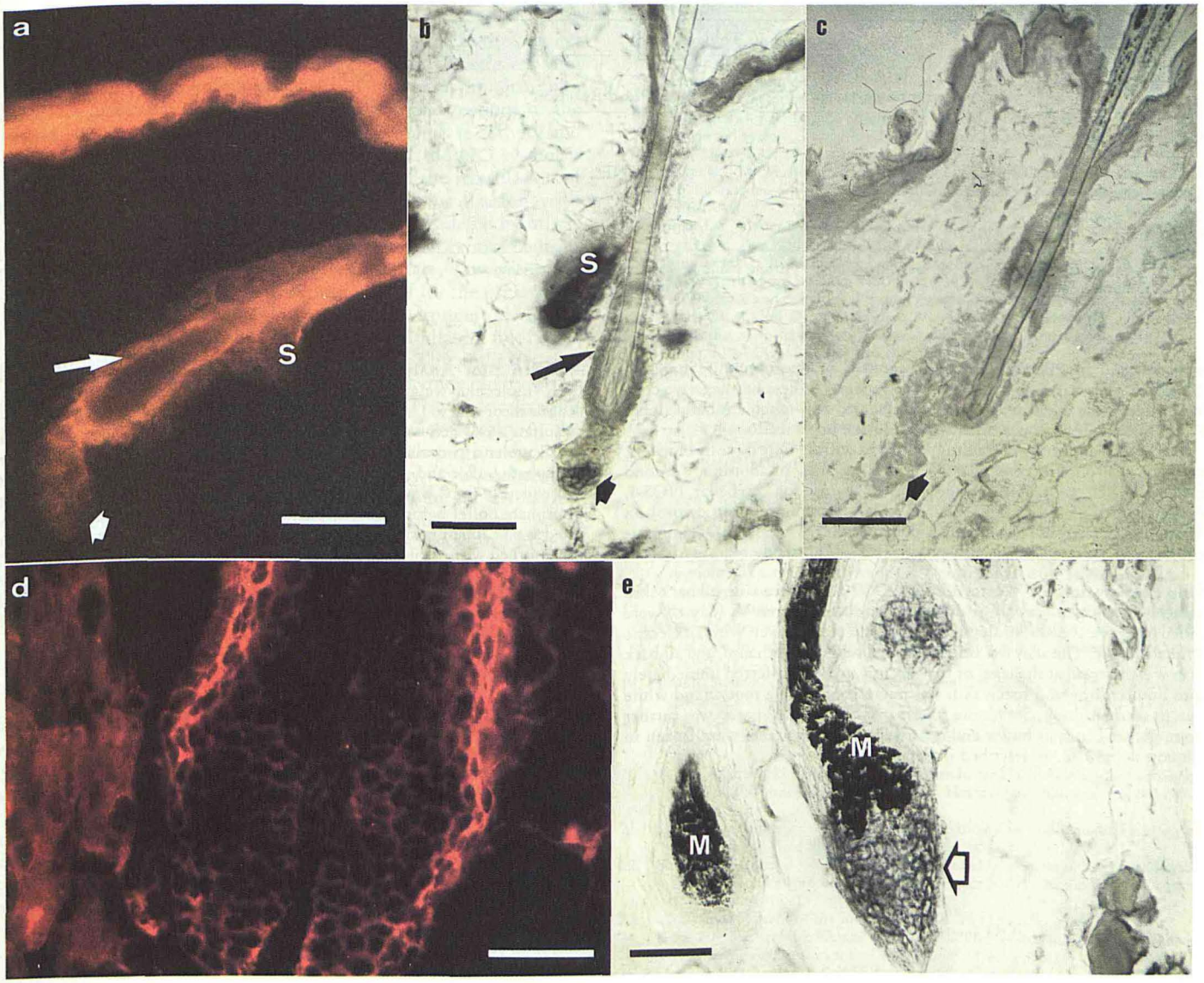

Figure 1. NOS-I immunohistology and NADPH-d histochemistry in murine skin. Tissues were examined with anti-NOS-I antibody (*a, d*) or assayed with TNBT (*b, c, e*) as described in *Methods. a,* Spontaneous telogen (day 0), with anti-NOS-I antibody revealing reactivity of the epidermis and in the region of the outer root sheath. Arrow (➡) indicates labeling of the outer root sheath. Sebaceous gland (S), dermal papilla (⇩). Bar, 50 μm. *b,* Cutaneous NADPH-d activity during telogen (day 0). Note the dark color reaction indicating NADPH-d activity, as assayed by TNBT, in epidermis, sebaceous gland, outer root sheath (➡) and dermal papilla (⇩). Sebaceous gland (S). Bar, 50 μm. *c,* Negative control of the NADPH-d reaction of the telogen follicle without NADPH. Dermal papilla (⇩). Bar, 50 μm. *d,* Depilation induced anagen VI (day 8). The anti-NOS-I antibody shows labeling in the region of the outer root sheath. Bar, 30 μm. *e,* Cutaneous NADPH-d activity during depilation induced anagen IV (day 8) of the murine hair cycle. Note the positive dark color reaction indicating NADPH-d activity of the proximal hair bulb (⇩). Melanin pigmentation (M). Bar, 50 μ.

described in detail, and full-thickness back skin was harvested either from untreated telogen mice, or from depilation-induced anagen mice that had just entered anagen VI (day 8 after depilation) [18,22]. Six- to eight-week-old female C 57 BL-6 mice were purchased from Charles River, Hannover, FRG, were housed in community cages at the FU Berlin Animal Facilities, UKRV, and were fed water and mouse chow *ad libitum.* All experiments reported below were done on 5-μm cryosections from at least three different mice per hair-cycle stage studied.

NADPH-Diaphorase Histochemistry Because NOS-I displays NADPH diaphorase activity (NADPH-d), this activity can be demonstrated by the reduction of the soluble dye tetranitroblue tetrazolium (TNBT) to the insoluble formazan TNBTF, which becomes visible on histology as a dark brown color reaction. The determination of NADPH-d activity is a widely accepted, though not fully selective, histochemical standard technique for detecting NOS-I activity[25–27].

NADPH-d staining was performed by incubating acetone-fixed cryostat sections mounted on poly-L-lysine covered glass slides with a solution containing 1 mM NADPH/0.2 mM TNBT/0.1 M Tris·HCL at pH 8.0 and 0.2% Triton X-100 for 30 min at 37°C, as described [25,27]. Murine brain sections served as positive controls because the brain displays very strong, selective NADPH-d activity corresponding to NOS-I antigen [24,25,28]. Negative controls were done by incubation of sections in the absence of either NADPH or TNBT. All reagents were purchased from Sigma, FRG.

NOS Immunohistology To compare the distribution of NADPH-d activity and NOS-I antigen, the immunoreactivity of skin cryosections was tested by immunofluorescence with a specific rabbit antiserum raised against the constitutive form (about 160 kDa protein) of the porcine cerebellar NOS [9,29,30]. Slides (5 μm) were air dried for 6 h and fixed by immersion in acetone at −20°C for 5 min. NOS-I immunoreactivity was visualized by immunofluorescence microscopy. Slides were preincubated with phosphate-buffered saline (PBS) containing 4% mouse serum for 30 min. After washing in PBS, slides were incubated with anti NOS-I (dilution 1 : 100) for 30 min at 25°C, washed in PBS (2 × 5 min), incubated with phythoerythrin (PE)–conjugated anti-rabbit antibody (dilution 1 : 50) for 30 min at 25°C,

Table I.

	Spontaneous Telogen Skin		Depilation-Induced Anagen VI Skin	
	NOS-I *Immunoreactivity*[a]	NADPH-d Reaction[a]	NOS-I Immunoreactivity	NADPH-d Reaction
Epidermis	+	+	+	+
Outer root sheath	+	+	+	+
Hair matrix	NE[b]	NE[b]	0	++
Dermal papilla	0	+	0	+++
Sebaceous gland	0	+++	0	+++
Sweat gland	+	+	+	+

[a] Staining intensity: 0, no staining; +, weak staining; ++, strong staining; +++, very strong staining.
[b] NE, not evaluable.

washed again in PBS (2 × 5 min), mounted in fluoromount (Shandon, Hamburg, Germany), and covered with glass coverslips. For negative controls, slides were incubated with PE-conjugated anti-rabbit antibody alone. Positive controls were performed with murine brain sections.

To check the specifity of staining, positive controls were done by blocking with purified porcine cerebellar NOS (0.2 μg/ml). In addition, a second specific polyclonal rabbit antibody raised against rat cerebellar NOS-I, which detects a single band (150-165 kDa) of crude rat brain cytosol on Western blot analysis [11], served as positive control for the NOS-I immunoreactivity detected by the anti-porcine NOS-I antibody reported here.

Skin Preparation for Western Blot C 57-BL-6 mice with all back skin follicles in telogen (day 0) or depilation-induced anagen VI (day 8) were killed by cervical dislocation under ether, and were shaven with an electric animal clipper. The skin was briefly washed with 70% ethanol, and all back skin was dissected at the level of the subcutis, to be transferred immediately into liquid nitrogen. Frozen skin was pulverized using a mortar and white quartz sand (Aldrich, Steinheim, Germany). Pulverized tissue was further homogenized in lysis buffer and centrifuged. Supernatants were frozen in aliquots at −80°C, as described in detail [22].

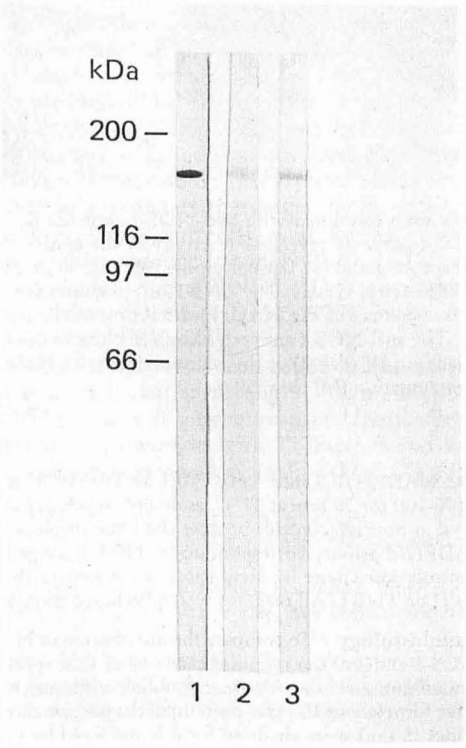

Figure 2. Western blot analysis of Typ I NOS with NOS-antiserum raised against the porcine cerebellar NOS-I. *Lane 1,* purified NOS-I from porcine cerebellum. *Lane 2,* homogenate of telogen mouse skin. *Lane 3,* homogenate of depilation-induced anagen skin. The position of the various molecular mass markers is indicated on the left.

Western Blot Analysis The samples (30 μg protein) and unstained, high-molecular-weight markers were separated under non-reducing conditions according to Laemmli [31]. Proteins were blotted from sodium dodecylsulfate (6%) gels to nitrocellulose membranes (Bio-Rad) for 60 min at 25°C under a constant current of 300 mA. Membranes were washed in phosphate buffer and saturated overnight with 5% fetal bovine serum and subsequently for 2 h with 3% bovine serum albumine and Tween 0.5% in phosphate buffer before the polyclonal anti-NOS rabbit serum [9] was applied at a 1 : 200 dilution. As negative control, the anti-NOS serum had been preabsorbed with adenosine diphosphate-eluted porcine NOS protein for 2 h at 25°C. After washing and incubation with anti-rabbit IgG-conjugated alkaline-phosphatase antibodies (Dianova, Hamburg, Germany), immune complexes were visualized by staining for alkaline-phosphatase activity and were photographed.

RESULTS

Cutaneous NADPH-diaphorase Activity During Anagen and Telogen C 57 Bl-6 mouse skin displayed strong and selectively distributed NADPH-d activity in the sebaceous glands (Fig 1*b*, Table I) and sweat glands (not shown) during both telogen and anagen. Weaker NADPH-d activity was detected in the epidermis and both the outer root sheath and the dermal papilla of the hair follicle (Fig 1*a*, Table I). NADPH-d activity was more marked in the dermal papilla during anagen VI (Table I). A less pronounced increase of NADPH-d activity during anagen was also noted in the hair matrix of the proximal hair bulb (Table I, Fig 1*e*). No significant and reproducible hair-cycle-dependent changes in NADPH-d activity were found in the other skin structures studied, such as epidermis or the outer root sheath (see Table I).

NOS-I Antigen Expression As detected with a specific antiserum against NOS-1, immunoreactivity of NOS-I antigen colocalized with NADPH-d activity in the epidermis, the outer root sheath of the hair follicle and the sweat glands (not shown) during telogen (Fig 1*a*; Table I). In contrast, no NOS-I immunoreactivity was seen in the sebaceous gland (Fig 1*a*; Table I). In the proximal hair bulb, only outer root sheath keratinocytes displayed NOS-I immunoreactivity, whereas in the dermal papilla and the hair matrix no immunoreactivity was seen (Fig 1*d*). In contrast, NADPH-d reactions stained the entire proximal hair bulb and the dermal papilla in anagen (Fig 1*e*). The increase of NADPH-d activity in the proximal hair bulb and the dermal papilla showed no comparative, substantial hair-cycle-dependent changes in NOS-I immunoreactivity. This pattern of immunoreactivity was confirmed with a different specific NOS-I antiserum [15] which showed labeling of the epidermis and the outer root sheath and of single cells in the dermis with morphologic characteristics of mast cells (not shown). Figure 2 shows the results of immunoblotting of skin homogenates of murine telogen and depilation-induced anagen VI skin, compared to NOS-I protein from purified porcine cerebellar NOS-I. An immunoreactive band of the skin homogenates was observed at the level of porcine NOS-I, all corresponding to a molecular weight of about 160 kDa. Increased immunoreactivity of NOS-I antigen was observed in anagen skin homogenates from all of three independent experiments.

DISCUSSION

NO and NOS activity have previously been studied in the context of their immunomodulatory, neurobiologic, and muscle-relaxing properties, yet little is known about this system in mammalian skin. We show here that normal mouse epidermis and sections of the epithelial hair bulb display both NADPH-diaphorase activity and NOS-I antigen. This raises the possibility that the NO system is involved in the control of skin physiology and the modulation of tissue growth and remodeling. Due to its co-localization with NOS-I antigen, NADPH-d activity has been considered by others to be a suitable marker for NOS-I in peripheral and central neurons [24–27]. In the present study, we demonstrate, however, that NADPH-I activity alone is a misleading marker for the presence of constitutive NOS-I in murine skin because the strongest NADPH-d activity in the sebaceous gland and dermal papilla did not correspond to detectible NOS-I immunoreactivity. This indicates that NADPH-d histochemistry may also detect enzyme activity other than NOS-I (e.g., dehydrogenases, [32–34]). The co-localization of both NADPH-d activity and NOS-I immunoreactivity in the epidermis and the outer root sheath of the follicle might nevertheless represent active NOS-I enzyme. Currently no histochemical method is available that allows us to prove that antigen displaying NOS-I immunoreactivity *in situ* has NOS enzyme activity.

It remains thus to be conclusively demonstrated that NOS-I antigen actually synthesizes NO in mammalian skin at the localization in the tissue suggested by immunohistochemistry in the present study. Our results support, nevertheless, the concept that nitrinergic signals play a role in cutaneous epithelium through paracrine secretion, as has been suggested for the regulation of bronchial [35], gastric [14,36], and endometrial epithelial perfusion [14].

Our study also suggests that murine skin has multiple potential cellular sources for NO production, i.e., cells equipped with the enzymatic machinery to generate NO. It remains a major challenge for future research to prove that NO synthesis actually occurs and that it is regulated during developmental processes *in vivo* under physiologic circumstances. Also, the functional significance of the NO system for skin physiology, specifically in the regulation of cell growth, differentiation, and tissue remodeling, remains to be dissected. In this context, it is noteworthy that Heck *et al* have recently shown that the inhibition of human and mouse keratinocyte proliferation by gamma-interferon can be reversed by co-culture with the NOS antagonist N-methyl-L-arginine [17].

The C 57 BL-6 mouse model offers a unique research tool for studying NO and NOS functions in a mature mammalian tissue interaction system. The present data suggest that changes in the level and activity of the enzyme might be present at different stages of the hair cycle. These findings will have to be confirmed by studying, for example, whether administration of NO-donating (nitroprussid, SIN-1) or NOS-inhibitory drugs (N-monomethyl-L-arginine, Nω-nitro-L-arginine) can alter murine hair growth *in vivo* or in a recently developed, complementary organ-culture assay [18,37,38]. Further studies will also have to exclude that the increased NOS-I immunoreactivity of anagen skin (Fig 2) simply reflects the significantly increased epithelial cell mass during the murine hair-cycle stages.

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