Regulation of Melanin Biosynthesis in the Human Epidermis by Tetrahydrobiopterin

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The participation of (6R) 5,6,7,8-tetrahydrobiopterin (6-BH_4) in regulating the tyrosine supply for melanin biosynthesis was investigated by the examination of human keratinocytes, melanocytes, and epidermal suction blisters from normal human skin and from patients with the depigmentation disorder vitiligo. Cells, as well as total epidermis, contained high phenylalanine hydroxylase activities and also displayed the capacity to synthesize and recycle 6-BH_4, the essential cofactor for this enzyme. In vitiligo, 4a-hydroxy-BH_4 dehydratase activity was extremely low or absent, yielding an accumulation of the nonenzymatic by-product 7-tetrahydrobiopterin (7-BH_4) at concentrations up to 8×10^{-6} M in the epidermis. This by-product is a potent competitive inhibitor in the phenylalanine hydroxylase reaction with an inhibition constant of 10^{-6} M. Thus, 6-BH_4 seems to control melanin biosynthesis in the human epidermis, whereas 7-BH_4 may initiate depigmentation in patients with vitiligo.

Tyrosine is a central metabolite in the human epidermis for the biosynthesis of the catecholamines by keratinocytes (*1*) and for the biosynthesis of the melanins in melanocytes (*2*). So far, tyrosinase [monophenol dihydroxyphenylalanine:oxygen oxidoreductase (E.C. 1.14.18.1)] has been assumed to be the key enzyme for melanin biosynthesis (*2*). The expression of tyrosinase in the human epidermis does not vary in different skin types (Fitzpatrick classification skin type I to skin type VI) (*2*). Tyrosinase messenger RNA shows no significant differences in black and white skin (*3*, *4*). Thus, melanin biosynthesis depends either on the substrate supply or on metabolic inhibitors and activators of this enzyme (*3*–*5*). The formation of L-tyrosine originates from L-phenylalanine by way of phenylalanine hydroxylase [L-phenylalanine–tetrahydropteridine oxygen oxidoreductase (E.C. 1.14.16.1)] and the essential cofactor (6R) 5,6,7,8 tetrahydrobiopterin (6-BH_4) (*6*).

In the presence of molecular oxygen, 6-BH_4 is the immediate electron donor for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Hence, 6-BH_4 also functions as an important cofactor in the synthesis of the neurotransmitters serotonin and the catecholamines (*7*). The de novo synthesis of 6-BH_4 begins with guanosine triphosphate (GTP) which is converted to D-erythro-7,8-dihydroneopterin triphosphate by GTP-cyclohydrolase I (E.C. 3.5.4.16) (GTP–CH-1) (*8*). The activity of this rate-limiting enzyme is constitutively expressed in liver and neuronal tissues. Dihydroneopterin triphosphate is converted to 6-pyruvoyl tetrahydropterin by its synthase, and the C1′ and C2′ oxo-functions are reduced to the final product 6-BH_4 by reduced nicotinamide adenine dinucleotide phosphate–dependent sepiapterin reductase (*7*). Therefore, the hydroxylation of aromatic amino acids is accomplished by a multi-enzyme system with 6-BH_4 as the end product. The metabolic pathway for the synthesis of L-tyrosine from L-phenylalanine by phenylalanine hydroxylase is presented in Fig. 1. In this cycle, phenylalanine hydroxylase and 4a-hydroxy-BH_4-dehydratase are closely synchronized for the regeneration of 6-BH_4 (*8*). The uncoupling of these two enzymes yields the production of hydrogen peroxide (*8*). It is proposed that this reaction occurs because of the instability of a 4a-peroxy-BH_4 intermediate yielding q-BH_2 and hydrogen peroxide as the products (*8*). In addition, on the basis of decreased 4a-hydroxy-BH_4 dehydratase activity, the metastable 4a-hydroxy-BH_4 substrate undergoes a nonenzymatic rearrangement with opening of the pyrazine ring followed by recyclization, yielding the 7-tetrahydrobiopterin isomer (7-BH_4) (*9*, *10*). This isomer competes for the 6-BH_4 binding site on phenylalanine hydroxylase, functioning as a potent competitive inhibitor (*10*, *11*). Recently, Harada and co-workers described a second control mechanism by identifying the presence of a feedback regulator protein (P-35) that is activated by L-phenylalanine and inhibited by 6-BH_4 in controlling GTP–CH-1 activity (Fig. 1) (*12*). The biosynthesis of the catecholamines and the melanins in the human epidermis occurs on a branched metabolic pathway with tyrosine as the common substrate, and 6-BH_4 controls the production of this amino acid. Thus, it was considered that the depigmentation disorder vitiligo could be used as a model system in understanding the control of pigmentation. Vitiligo is an acquired depigmentation disorder affecting 0.5 to 4% of the world's population. Both slow and rapid progress have been reported as well as stable disease and spontaneous remission (*13*). Melanocytes may be present in the depigmented epidermis, but they are unable to produce pigment. The etiology of this disease remains an enigma.

Both human undifferentiated control keratinocytes and control melanocytes can synthesize 6-BH_4 from GTP and contain significant phenylalanine hydroxylase activity (Table 1) (*14*–*17*). Differentiated keratinocytes contain lesser amounts of biopterin and the three enzymes studied. The metabolic pathway for the biosynthesis of L-tyrosine is down-regulated after cell differentiation. Keratinocytes established in cultures from both lesional and nonlesional

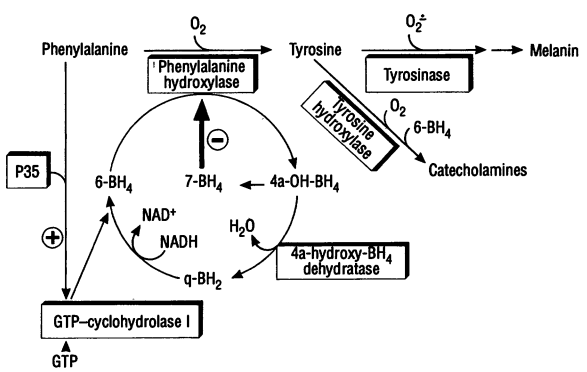

Fig. 1. Scheme for the de novo biosynthesis and recycling of 6-BH_4 in the regulation of L-tyrosine production by phenylalanine hydroxylase in the human epidermis. L-Tyrosine is the common substrate for melanin biosynthesis by melanocytes and catecholamine biosynthesis by keratinocytes. In vitiligo, it is proposed that the low activity for 4a-hydroxy-BH_4 dehydratase leads to a buildup of 7-BH_4 in the epidermis. As a consequence of this buildup, phenylalanine hydroxylase is inhibited. Phenylalanine accumulates and, by way of the feedback regulator P35, induces GTP–CH-1. This latter enzyme is the rate-limiting step for 6-BH_4 synthesis and, therefore, more 7-BH_4 is produced, causing depigmentation.

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skin in vitiligo contained significantly elevated levels of biopterin (Table 1), but expressed lower (30 to 60%) 4a-hydroxy-BH_4 dehydratase activities compared to those of controls (Fig. 2). Melanocytes from healthy individuals and from one patient with vitiligo confirmed that 4a-hydroxy-BH_4 dehydratase activity is negligible in vitiliginous cells (Fig. 2). GTP–CH-1 activity from nonlesional skin appears to be higher than in controls (Table 1).

The results for total epidermal extracts from healthy volunteers with fair and dark skin and from three patients with active vitiligo are summarized in Table 2. The total biopterin concentration in the human epidermis increases with pigmentation, but vitiliginous epidermis contains extremely high concentrations of this cofactor. In active vitiligo, the isomer 7-biopterin approaches a cellular concentration of 8×10^{-6} M [for calculation, see (*15*) and notes therein], whereas the GTP–CH-1 activity is increased from 3- to 10-fold (Table 2). However, phenylalanine hydroxylase activities are low, and 4a-hydroxy-BH_4 dehydratase activities are barely detectable (Table 2 and Fig. 2). This high concentration of 7-BH_4 in vitiliginous epidermal extracts suggested an inhibitory function for 7-BH_4 in phenylalanine hydroxylase activity. To test this point, phenylalanine hydroxylase activity was determined in vitiliginous cell extract (patient 3, Table 2). After the total biopterin was removed from this extract by gel filtration chromatography, the specific activity of phenylalanine hydroxylase increased from 2.4 to 16.5 nmol mg^{-1} min^{-1}. Upon the addition of 4.0 μmol of 7-BH_4 to the fully activated extracts, the specific activity was reduced from 16.5 to 5.0 nmol mg^{-1} min^{-1} (Fig. 3A). The cell-free extracts used in this experiment (Fig. 3A) contained 81 pmol of 7-biopterin per milligram of protein, yielding a cellular concentration of 8.0 μM. To test if this inhibition applies for melanin biosynthesis in normal melanocytes and normal skin, a concentration-dependent inhibition of phenylalanine hydroxylase by 7-BH_4 in cell extracts from melanocytes and from epidermal suction blisters from black skin was performed (Fig. 3B). Apparent inhibition constant (K_I) values of 1.2×10^{-6} M and 2.53×10^{-6} M, respectively, were determined, verifying 7-BH_4 as an inhibitor of phenylalanine hydroxylase in the skin. The accumulation of 7-BH_4 and its oxidation to 7-biopterin were confirmed in vivo by comparison of the fluorescence emission spectrum in cell extracts prepared from the epidermis of lesional skin and from vitiliginous keratinocytes compared to that of controls. The depigmented fluorescent skin, together with normal pigmented skin, on the leg of a patient with active vitiligo after exposure to WOOD's light (ultraviolet wavelengths of 320 to 400 nm, low-pressure lamp) is shown in Fig. 4. The fluorescence is quenched by the melanin in the normal skin.

Our results show that the entire meta-

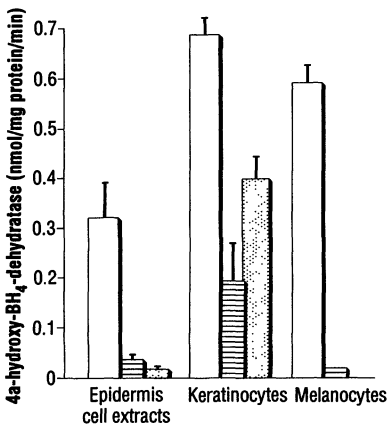

Fig. 2. Activities specific to 4a-hydroxy-BH_4 dehydratase in extracts from epidermal suction blisters (vitiligo, $n = 7$; controls, $n = 3$) and from normal keratinocytes ($n = 3$), vitiliginous keratinocytes ($n = 3$), nonlesional keratinocytes ($n = 3$), normal melanocytes ($n = 3$), and vitiliginous melanocytes ($n = 1$). Open bars, control; striped bars, vitiligo lesional; dotted bars, vitiligo nonlesional. The histogram presents mean values with the mean of standard deviations. 4a-hydroxy-BH_4 dehydratase activity was measured in a coupled assay to phenylalanine hydroxylase, in which the dehydratase served as the rate-limiting enzyme for the recycling of catalytic amounts of 6-BH_4 (*17*).

Table 1. Human keratinocytes were established from epidermal suction blister roofs of vitiliginous and nonlesional skin of a patient with vitiligo and from age-matched controls of the same skin type (*3, 14*). Melanocytes were established from human foreskin (*14*). Cell extracts were prepared from these cells, and total biopterin as well as the 6 and 7 isomers were determined with reversed-phase HPLC (*15*). GTP–CH-1 activity was measured as neopterin and neopterin di- and triphosphate production from GTP with ion-pairing HPLC (*16*). Phenylalanine hydroxylase activity was determined following the production of [^{14}C](U)–labeled L-tyrosine from [^{14}C]U–labeled L-phenylalanine (*17*). All assays were performed in duplicate (mean error < 2%). GTP–CH-1 activity was close to the detection limit of 0.15 to 0.20 pmol per milligram of protein per hour. The 6- and 7-biopterins were below the limits for accurate determination in keratinocytes (n.d.) that were used for the table.

Cell type	Total biopterin (pmol/mg of protein)	6-biopterin (pmol/mg of protein)	7-biopterin (pmol/mg of protein)	GTP cyclohydrolase I (pmol/mg of protein/hour)	Phenylalanine hydroxylase (nmol/mg of protein/min)
Melanocytes (control)	25.5	17.2	8.3	3.3	3.2
Keratinocytes (control)	5.97	n.d.	n.d.	0.17	7.99
Keratinocytes vitiligo (lesional)	41.12	39.08	2.04	0.24	7.87
Keratinocytes vitiligo (nonlesional)	146.25	135.55	10.7	0.55	6.99

Table 2. Cell cultures from human suction blister roofs from normal healthy epidermis of four volunteers with skin types I and II (Caucasian), IV (Native American), and VI (African American) according to the Fitzpatrick classification (*3*) were used in these experiments and compared to the lesional (depigmented) and nonlesional (pigmented) epidermis of three patients with active vitiligo (skin types II, IV, and VI) [the methods are described in Table 1 and (*15–17*)].

Skin type	Total biopterin (pmol/mg of protein)	6-biopterin (pmol/mg of protein)	7-biopterin (pmol/mg of protein)	GTP cyclohydrolase I (pmol/mg of protein/hour)	Phenylalanine hydroxylase (nmol/mg of protein/min)
I	13.5	9.7	3.8	1.6	4.2
II	18.7	11.8	6.9	3.04	7.8
IV	32.6	22.6	10.0	2.9	17.9
VI	76.0	65.0	11.0	2.5	26.4
II					
Vitiligo lesional	551	468	83	15.9	3.2
Vitiligo nonlesional	953	874	79	26.1	4.2
IV					
Vitiligo lesional	186	97	89	11.1	0.1
Vitiligo nonlesional	218	154	64	13.5	4.2
VI					
Vitiligo lesional	223	142	81	32.1	2.30
Vitiligo nonlesional	256	185	71	31.6	2.14

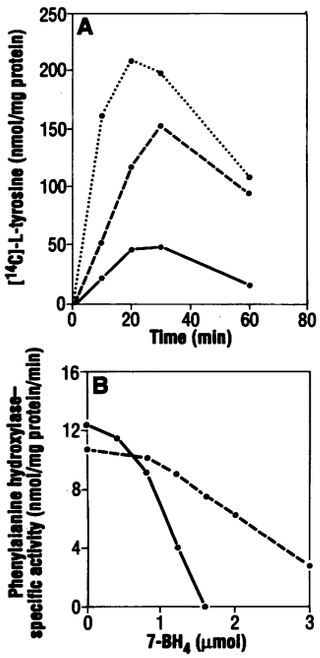

Fig. 3. (**A**) Phenylalanine hydroxylase activities under saturating cofactor and substrate conditions in epidermal extracts of a patient with active vitiligo. The decrease in L-tyrosine formation between 30 and 60 min is most likely due to the activity of tyrosinase in these crude extracts. Bottom trace, crude extract from lesional epidermis; top trace, after chromatography of the lesional epidermis from the bottom trace; middle trace, after the addition of 4.0 μmol of 7-BH_4 to the top trace. (**B**) The inhibition of phenylalanine hydroxylase (*17*) activity by 0×10^{-6} M to 3×10^{-6} M 7-BH_4 in extracts from (solid trace) human melanocytes and (dashed trace) black epidermis (skin type VI).

bolic pathway for L-tyrosine biosynthesis occurs in both melanocytes and undifferentiated keratinocytes with activities comparable to those reported for liver (8, 9), whereas differentiated keratinocytes from normal subjects yield extremely low activities. As a consequence, the majority of tyrosine production appears to be located under normal healthy conditions only in the basal layer of the epidermis where melanocytes and undifferentiated keratinocytes coexist, forming the epidermal unit (*2*). The tyrosine supply is controlled by GTP–CH-1, the rate-limiting enzyme for the synthesis of 6-BH_4, and phenylalanine. The activities of the tyrosine biosynthetic pathway correspond with human skin pigmentation, yielding significantly more phenylalanine hydroxylase activity in dark as compared to white skin (Table 2). In patients with vitiligo, the cycling of 6-BH_4 is impaired, leading to a time-dependent accumulation of 7-BH_4 (Fig. 1). The presence of this nonenzymatic by-product in the epidermis may initiate the process of depigmentation by blocking the supply of L-tyrosine either directly in the melanocytes

Fig. 4. The fluorescence of the depigmented epidermis on the leg of a patient with active vitiligo is shown compared to aqueous solutions of 6- and 7-biopterin. In the adjacent pigmented skin, the fluorescence is quenched by the melanins. Confirmation of the in vivo existence of 6- and 7-biopterins was obtained by the measurement of the excitation spectrum of epidermal cell extracts from lesional skin and vitiliginous keratinocytes with the use of fluorescence spectroscopy. In vivo 6- and 7-biopterins represent the principle fluorescent molecules in these patients. Excitation with 320- to 400-nm WOOD's light has been used as the major diagnostic tool for vitiligo by dermatologists. Depigmentation of the other origin does not show this characteristic fluorescence.

or from the surrounding basal keratinocytes in the skin of patients with vitiligo. Finally, only patients with active depigmentation accumulate sufficient 7-BH_4 to cause the striking fluorescence of the lesional skin observed upon exposure to ultraviolet light (Fig. 4), whereas stable disease does not show this characteristic pattern.

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