Epidermal H_2O_2 Accumulation Alters Tetrahydrobiopterin ($6BH_4$) Recycling in Vitiligo: Identification of a General Mechanism in Regulation of All $6BH_4$-Dependent Processes?

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It has been shown *in vivo* that patients with the depigmentation disorder vitiligo accumulate hydrogen peroxide (H_2O_2) accompanied by low catalase levels and high concentrations of 6- and 7-biopterin in their epidermis. Earlier it was demonstrated that epidermal 4a-OH-tetrahydrobiopterin dehydratase, an important enzyme in the recycling process of 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$), has extremely low activities in these patients concomitant with a build-up of the abiogenic 7-isomer ($7BH_4$), leading to competitive inhibition of epidermal phenylalanine hydroxylase. A topical substitution for the impaired epidermal catalase with a pseudocatalase effectively removes epidermal H_2O_2, yielding a recovery of epidermal 4a-OH-tetrahydrobiopterin dehydratase activities and physiologic $7BH_4$ levels in association with successful repigmentation demonstrating recovery of the $6BH_4$ recycling process. Examination of recombinant enzyme activities, together with 4a-OH-tetrahydrobiopterin dehydratase expression in the epidermis of untreated patients, identifies H_2O_2-induced inactivation of this enzyme. These results are in agreement with analysis of genomic DNA from these patients yielding only wild-type sequences for 4a-OH-tetrahydrobiopterin dehydratase and therefore ruling out the previously suspected involvement of this gene. Furthermore, our data show for the first time direct H_2O_2 inactivation of the important $6BH_4$ recycling process. Based on this observation, we suggest that H_2O_2 derived from various sources could be a general mechanism in the regulation of all $6BH_4$-dependent processes.
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Patients affected with the depigmentation disorder vitiligo express a characteristic fluorescence in their white skin patches upon Wood's light (351 nm) examination (Schallreuter *et al*, 1994a, b). In this context, it was demonstrated that this phenomenon originates from the accumulation of oxidized pterins (Schallreuter *et al*, 1994a, b). In the presence of molecular oxygen 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) is the immediate electron donor for the hydroxylation of the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan (Kaufman and Fisher, 1974). Hence, $6BH_4$ functions as an important regulator in the synthesis of the neurotransmitters serotonin (Friedman *et al*, 1972) and catecholamines (Nagatsu *et al*, 1964). L-tyrosine is the central substrate for both human epidermal undifferentiated keratinocytes and melanocytes to initiate catecholamine synthesis and melanogenesis in these cells. Under normal physiologic conditions, both cell types hold the full capacity for *de novo* synthesis/recycling/regulation of $6BH_4$ (Schallreuter *et al*, 1994a, b). It has also been demonstrated that $6BH_4$ can regulate tyrosinase (EC 1.14.18.1) by uncompetitive inhibition (Wood *et al*, 1995). **Figure 1** presents a scheme for the *de novo* synthesis/recycling/regulation of $6BH_4$ and its link to melanocytes and keratinocytes. In this context, it was realized that patients with vitiligo show an increased epidermal *de novo* synthesis and recycling of this cofactor in association with barely detectable 4a-OH-tetrahydrobiopterin dehydratase (DH; EC 4.2.1.96) activities (synonyms: pterin 4 alpha carbinolamine dehydratase (PCD), dimerization cofactor of hepatocyte nuclear factor 1 α (DCoH)) (Schallreuter *et al*, 1994a; 1994b). This enzyme is of major importance in the recycling process of $6BH_4$ (Kaufman, 1970; Huang *et al*, 1973; Ayling *et al*, 1997) (**Fig 1**). Low activities of DH can lead to the accumulation of the abiogenic 7-isomer ($7BH_4$) (Curtius *et al*, 1990; Davis *et al*, 1991; Adler *et al*, 1992), which is a potent competitor of the natural cofactor $6BH_4$, hence severely affecting phenylalanine hydroxylase (PAH; EC 1.14.16.1) activities (Davis *et al*, 1992). Decreased PAH activities were indeed demonstrated in the skin of patients with vitiligo (Schallreuter *et al*, 1994a, b). One consequence of low

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Abbreviations: $6BH_4$, 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin; $7BH_4$, abiogenic 7-isomer; DH, 4a-OH-tetrahydrobiopterin dehydratase; FT-Raman spectroscopy, Fourier transform Raman spectroscopy; PAH, phenylalanine hydroxylase.

[1]In this study we used the pseudocatalase formulation (PC-KUS).

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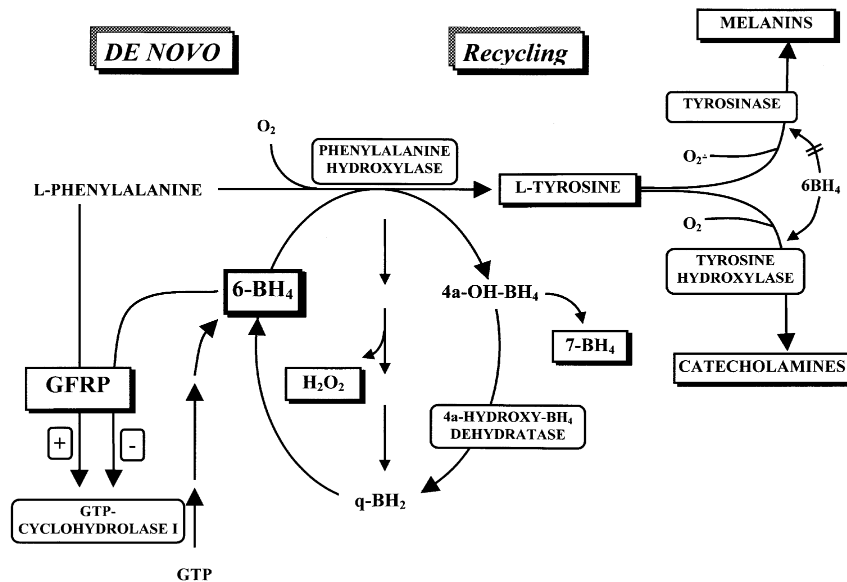

Figure 1. *De novo* synthesis/recycling/regulation of $6BH_4$ in the production of L-tyrosine by PAH as the substrate for melanogenesis and catecholamine synthesis. Both epidermal keratinocytes and melanocytes hold the full capacity for an autocrine *de novo* production/regulation and recycling of $6BH_4$ (Schallreuter *et al*, 1994a, b). Guanosine triphosphate cyclohydrolase I (GTP-CHI) is the rate limiting step in *de novo* synthesis of $6BH_4$ using GTP as the starting substrate. $6BH_4$ serves as the cofactor for the hydroxylation of the essential amino acid L-phenylalanine to L-tyrosine (Kaufman and Fisher, 1974). L-tyrosine is the substrate for tyrosinase to initiate melanogenesis in melanocytes. It is also the substrate for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis in keratinocytes (Schallreuter *et al*, 1992). Tyrosinase is regulated by $6BH_4$ through uncompetitive inhibition, whereas tyrosine hydroxylase requires $6BH_4$ as a cofactor for the hydroxylation reaction of L-tyrosine to L-DOPA. In addition, PAH produces 4a-OH-BH_4 as an intermediate in the recycling process of $6BH_4$, which is dehydrated by DH to quinonoid dihydropterin (qBH_2) followed by the NADPH-dependent reduction back to $6BH_4$. During the recycling, the 7-isomer ($7BH_4$), is nonenzymatically produced from 4a-OH-BH_4 (Curtius *et al*, 1990; Davis *et al*, 1991). $7BH_4$ can inhibit PAH, as observed in untreated vitiligo (Davis *et al*, 1992; Schallreuter *et al*, 1994a). Consequently, the short circuit to qBH_2 causes the formation of H_2O_2 from O_2 and $6BH_4$ (Kaufman and Fisher, 1974). The *de novo* synthesis is controlled by the GTP-CHI feedback regulatory protein (GFRP), where L-phenylalanine upregulates via GFRP the activity of GTP-CHI and $6BH_4$ exerts the opposite effect (Harada *et al*, 1993; Milstien *et al*, 1996).

epidermal PAH activities in these patients is an increase in epidermal L-phenylalanine levels (Schallreuter *et al*, 1994a, b; , 1998), which has been confirmed by *in vivo* Fourier transform Raman spectroscopy (FT-Raman spectroscopy). This amino acid resolves as a well-defined peak at $1004\,cm^{-1}$ (Schallreuter *et al*, 1998). Both the epidermal L-phenylalanine and $7BH_4$ build-up, together with decreased PAH and DH activities, lead to the generation of hydrogen peroxide (H_2O_2) in the skin due to an increased short circuit in the recycling process of $6BH_4$ (Davis *et al*, 1992; Schallreuter *et al*, 1999) (**Fig 1**). To date several other impaired metabolic steps have been identified in this disorder yielding an accumulation of H_2O_2 in the epidermal compartment (Schallreuter *et al*, 1996; 1999; Maresca *et al*, 1997). On the one hand, it was only recently that *in vivo* noninvasive FT-Raman measurements on the skin of this patient group confirmed high concentrations of H_2O_2 (Schallreuter *et al*, 1999). On the other hand, accumulation of H_2O_2 can deactivate catalase (Aronoff, 1965). In this context, low catalase levels, as well as low glutathione peroxidase levels, have been identified in patients with vitiligo (Schallreuter *et al*, 1991; Maresca *et al*, 1997; Beazley *et al*, 1999). Moreover, these results are in agreement with various degrees of vacuolation/lipid peroxidation in epidermal cells of vitiligo skin biopsies described by many groups (Moellmann *et al*, 1982; Bhawan and Bhutani, 1983; Gokhale and Mehta, 1983; Tobin *et al*, 2000). H_2O_2 accumulation can oxidize $6BH_4$ as well as $7BH_4$ (Armarego *et al*, 1983; Armarego, 1984; Schallreuter *et al*, 1999) and 6-biopterin is cytotoxic to melanocytes (Schallreuter *et al*, 1994c). Here, it is noteworthy that both oxidation products can explain the characteristic fluorescence of the depigmented/white skin in these patients (Schallreuter *et al*, 1994a, b). This fundamental scientific observation led to the development of a pseudocatalase as a substitution for the impaired H_2O_2 degrading systems (Schallreuter *et al*, 1995, 1999). The topical application of this narrow band (311 nm) ultraviolet B (UVB) activated complex yielded four fundamental clinical results: (i) the arrest of an active depigmentation process in 95% of the patients (Schallreuter *et al*, 1995, 1999); (ii) the recovery of epidermal cells from intracellular vacuolation (Schallreuter *et al*, 1999; Tobin *et al*, 2000); (iii) a successful repigmentation in 60%–65% of all patients treated so far ($n > 700$); and (iv) initiation of repigmentation independent of the duration of the disease (Schallreuter *et al*, 1995, 1999). Until now, however, it remained unknown whether H_2O_2 accumulation could directly affect pterin homeostasis in the epidermis. We therefore embarked on a detailed analysis of the $6BH_4$ *de novo* synthesis and recycling with special emphasis on DH enzyme activity and on DH protein expression.

Our results identified restored epidermal DH activities in association with normal $7BH_4$ levels in patients treated with a topical pseudocatalase. This observation was accompanied by a significant decrease/complete loss of epidermal H_2O_2 confirmed *in vivo* by FT-Raman spectroscopy. The validity of direct H_2O_2 deactivation of DH has been proven by experiments with pure recombinant enzyme, as well as with immunohistochemical analysis of skin sections from treated and untreated patients. Computer modeling identified a structural change of the enzyme active site of DH after H_2O_2 oxidation of the cys^{81} and trp^{24} residues. All of the above observations were supported in patients with vitiligo by an examination of genomic DH to rule out a suspected point mutation of this important enzyme. Only wild-type sequences were detected in the DH coding region in all cases studied. The observations from this study indicate a general mechanism for H_2O_2-impaired $6BH_4$ recycling in this disorder.

MATERIALS AND METHODS

Patients Two independent patient groups and ten healthy age and sex matched controls served for this investigation. Both studies were approved by the local Ethics Committee and patient consent forms were obtained from each patient and control prior to the experiments. All patients and controls had photo skin type III (Fitzpatrick classification) (Fitzpatrick *et al*, 1971). The patients expressed the common type vitiligo vulgaris (mean age 50.9 y; range 19–66 y), and the mean duration of the disease at the point of investigation was 18 y (range 2–44 y). All patients were otherwise healthy and did not take any additional medication or cosmetics. The first group included 13 patients for the analysis of pterins and enzyme activities before and after 6 mo treatment with a UVB-activated pseudocatalase (Schallreuter, 1999; Schallreuter *et al*, 1999). The second group included 15 patients for the *in vivo* FT-Raman measurements of epidermal H_2O_2 before treatment and 13 d after treatment with a UVB-activated pseudocatalase.

Cell cultures Human epidermal melanocytes and keratinocytes from normal healthy probands and patients with vitiligo were established from suction blister material in MCDB 153 medium using the method of Wille *et al* (1984).

Epidermal cell extracts and protein determination Cell extracts were produced from suction blister roofs using the method of Kiistala (1968). Protein content of each sample was determined employing the method of Kalb and Bernlohr (1977).

Analysis of epidermal pterins Total biopterin content was determined in epidermal cell extracts obtained from suction blister material prior to any treatment and 6 mo after treatment with pseudocatalase after acidic iodine oxidation using reverse phase high performance liquid chromatography (HPLC) following the method from Ziegler and Hültner (1992). The concentration of pterin was calculated on the basis of protein content (Kalb and Bernlohr, 1977).

PAH activities in epidermal cell extracts PAH activities were measured in the same epidermal cell extracts as were used for analysis of pterins using the method of Abita *et al* (1987; Schallreuter *et al*, 1994a). Enzyme activities were determined per mg protein per min.

DH activities in epidermal cell extracts Enzyme activities were determined in the same epidermal cell extracts as above in a coupled assay according to Schallreuter *et al* (1994a).

DH mRNA expression in normal and vitiliginous melanocytes and keratinocytes Total mRNA was extracted from cells prior to the fourth passage using Ambions Totally RNA isolation kit (AMS Biotechnology, Oxfordshire, U.K.) based on the guanidium isothiocyanate method. cDNA was synthesized using the reverse transcription system (Promega, Southampton, U.K.) and oligo DT primers, incubated at 42°C for 1 h, followed by a 5 min denaturation step at 95°C. Polymerase chain reaction (PCR) assays were conducted as described previously (Schallreuter *et al*, 1998).

All primers were obtained from Genoysis Biotechnologies, Europe Ltd, Pampisford, U.K., and were designed based upon the mRNA sequences of Genbank accession numbers: DH (L41559), 5′-GCCATCTTCAAG-CAGTTTC-3′ and 5′-AGTTCAGTCACCCTTTCCC-3′; β-actin (M10277); 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. Primers were added to final concentrations of 1.0 μm and 0.4 μm for DH and β-actin, respectively. DH reactions contained 10% dimethyl sulfoxide and all assays were subjected to incubations of 95°C for 5 min followed by 35 cycles of 95°C 1 min, 55°C 1 min, 72°C 1 min, and a final extension period of 72°C 11 min. PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining.

Immunohistochemistry Full-skin biopsies were obtained under local anesthesia from healthy controls of photo skin type III ($n = 8$) and from lesional white and uninvolved skin of patients with vitiligo ($n = 10$) with the same photo skin type. Samples were frozen in liquid nitrogen after cryoprotection. Seven-micron cryosections were allowed to air dry and were fixed in acetone for 10 min. Briefly, sections were incubated for 90 min at room temperature with the primary polyclonal DH antibody F 3862 (Resibois *et al*, 1999). Subsequently the sections were incubated for 20 min with a biotinylated secondary antibody followed by peroxidase labeled acridine (Dako, Carpinteria, CA). AEC was applied as chromogen (large volume Dako AEC substrate system) under microscopic control with a Leica DM IRB inverted microscope (Wetzlar, Germany) until the specific staining was visible. For embedding, glycergel mounting medium (Sigma-Aldrich, Dorset, U.K.) was used. For negative controls the primary antibody was omitted.

Transformation and purification of rat recombinant DH His-tagged rat recombinant DH was transformed into *Epicurian coli* BL21 (DE3) cells by using a PACYC 9d kanamycin-resistant vector according to the method of Ficner *et al* (1995). DH was overexpressed with 2×10^{-3} M isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 4 h and DH was partially purified by elution from DEAE-sepharose (1.5 × 10 cm) using a 100 ml gradient of 0.05–0.5 M NaCl in Tris buffer 0.05 M pH 8.0. DH fractions were then added to a Hi-Trap His-tagged affinity column (5.0 ml) loaded with Ni^{2+} and eluted by using a 100 ml gradient of 0.005–1 M imidazole buffer pH 8.0. DH purity was confirmed by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis. DH migrates as a single band at 14 kDa (data not shown). Note that the sequences of rat and human DH are identical (Ficner *et al*, 1995).

DH mutation analysis in nine patients with vitiligo Genomic DNA was isolated from epidermal sheets of full-skin biopsies and from blood leukocytes following standard procedures (Sambrook *et al*, 1989). Mutation analysis was performed by exon-specific amplification and direct DNA sequencing of the PCR products. The corresponding primer sequences for PCR and DNA sequencing have been published elsewhere (Thöny *et al*, 1998).

***In vivo* detection of H_2O_2 in the epidermis using FT-Raman spectroscopy** FT-Raman spectra were produced with a Bruker RFS 100/S spectrometer equipped with a liquid nitrogen cooled germanium detector. Sample excitation was accomplished using an Nd^{3+}:YAG laser operating at 1064 nm. The laser power was 400 mW. Each spectrum was accumulated from the wrist area over 5 min with 300 scans and a resolution of 4 cm^{-1}. Total H_2O_2 was assigned as a well-defined peak at 875 cm^{-1} based on the O–O stretch (Schallreuter *et al*, 1999a). Levels are expressed in arbitary units. *In vitro* experiments show that millimolar concentrations are required to follow a well-resolved peak at 875 cm^{-1} (Schallreuter *et al*, 1999a).

Molecular modeling based on the crystal structure of DH All molecular models were based on the crystal structure of PCD/DCoH (Protein Databank accession number 1DCP) (Cronk *et al*, 1996). For studies of the active site, a molecular model of the natural substrate 4a-OH-BH$_4$ was produced with the Hyperchem^{TM} suite of programs (Hypercube, Canada). Geometry optimization was obtained using the MM+ forcefield (Allinger, 1977). Both stereo isomers of 4a-OH-BH$_4$ were modeled and substituted in the same location as the product analog. In addition, selected residues were oxidized to mimic the effects of exposure to H_2O_2. All protein structures were optimized with the AMBER forcefield using a Polak-Ribiere conjugate gradient on a Pentium II personal computer running at 300 MHz with 64 Mb of memory (Weiner *et al*, 1986). Structures were visualized employing Chemscape Chime version 2.03 (MDL Information Systems, San Leandro, CA).

Statistical analysis Statistical analysis was based on the Student's paired *t* test on SPSS for Windows.

RESULTS

Presence of millimolar H_2O_2 concentrations in the epidermis of patients with vitiligo and its removal by a pseudocatalase The results of *in vivo* FT-Raman spectroscopy confirmed millimolar H_2O_2 accumulation in 15 untreated patients with active, progressive vitiligo. H_2O_2 was successfully removed after 13 d treatment with a topical application of a pseudocatalase (**Fig 2**).

Recovery of epidermal DH activities and 7BH$_4$ levels We therefore determined both DH activities as well as 7BH$_4$ levels in epidermal suction blister material obtained from patients before and 6 mo after initiation of the treatment. The results of all treated patients showed a restoration of DH activities concomitant with normal levels of 7BH$_4$ (**Figs 3, 4**). This observation clearly

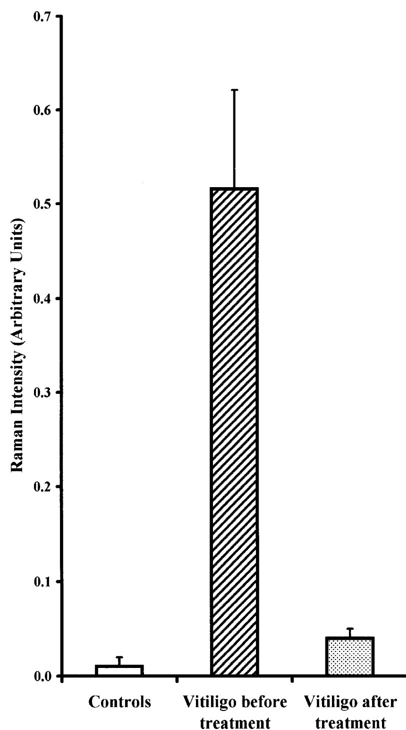

Figure 2. *In vivo* FT-Raman analysis of epidermal H_2O_2. *In vivo* H_2O_2 levels in white skin of patients with vitiligo ($n = 15$) compared with healthy controls ($n = 15$) were obtained before and after 13 d of treatment with pseudocatalase. These data prove the complete reduction of epidermal H_2O_2 after treatment with pseudocatalase ($p < 0.0001 \pm$ SEM).

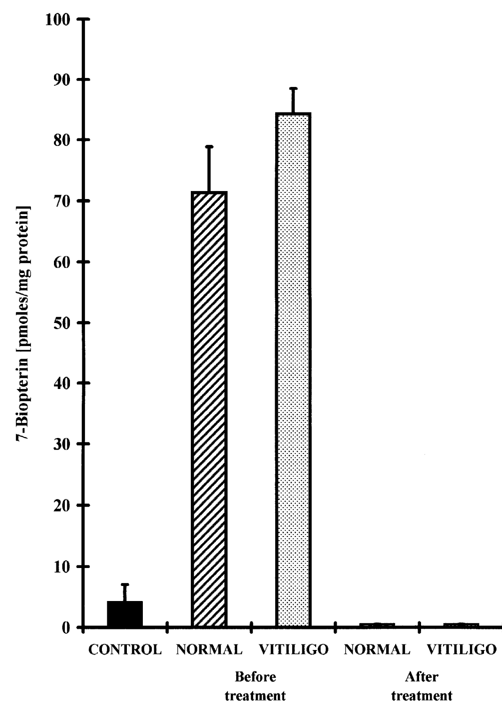

Figure 3. Epidermal $7BH_4$ levels before and after 6 mo treatment with pseudocatalase compared with healthy controls. The results demonstrate the recovery of pathologic to normal/physiologic levels of $7BH_4$ in patients after removal of H_2O_2 with pseudocatalase ($n = 10$) (error bars show standard error of the mean) ($p < 0.0001 \pm$ SEM) (normal = nonlesional, vitiligo = lesional).

indicates the recovery of the recycling process for $6BH_4$ after removal of excessive H_2O_2 in the epidermis of these patients. Enzyme activities and pterin levels, however, are determined on the basis of protein content. Therefore, this value is influenced by the thickness of the stratum corneum, which does not express any enzyme activities. Highest activities are always found in proliferating cells (i.e., the basal layer) with a significant reduction after differentiation (Schallreuter *et al*, 1994a; 1994b). Note that after 6 mo treatment the stratum corneum is always thicker and therefore total DH activities probably fully recover. This assumption is supported by the full recovery of $7BH_4$ production in these patients. Here it should be noted that significant $7BH_4$ levels are only produced when DH is absent or inhibited (Curtius *et al*, 1988; Davis *et al*, 1991).

Recovery of DH protein in the epidermis after H_2O_2 removal In addition to the recovery of DH enzyme activities, we documented normal expression of DH immunoreactivity using a polyclonal antibody F 3862 against DH. **Figure 5** demonstrates a significantly higher expression of DH in the basal layer of the epidermis in both patients and controls. This observation is in agreement with upregulated proliferation in these cells concomitant with autocrine $6BH_4$ production/recycling/regulation and catecholamine biosynthesis (Schallreuter *et al*, 1992; Schallreuter, 1997). Untreated patients show a much lower expression of DH protein compared with treated patients and healthy controls of the same photo skin type (Fitzpatrick classification) (**Fig 5*a–c***). These results demonstrate a decrease in protein levels of the enzyme in the untreated patients in association with epidermal accumulation of H_2O_2 (**Fig 2**). As the expression of DH mRNA from epidermal melanocytes and keratinocytes (**Fig 6**) appeared to be unaffected compared with control cells, it can be suggested that the decrease in the DH protein as shown in **Fig 5(*b*)** could be directly initiated by H_2O_2 due to protein instability.

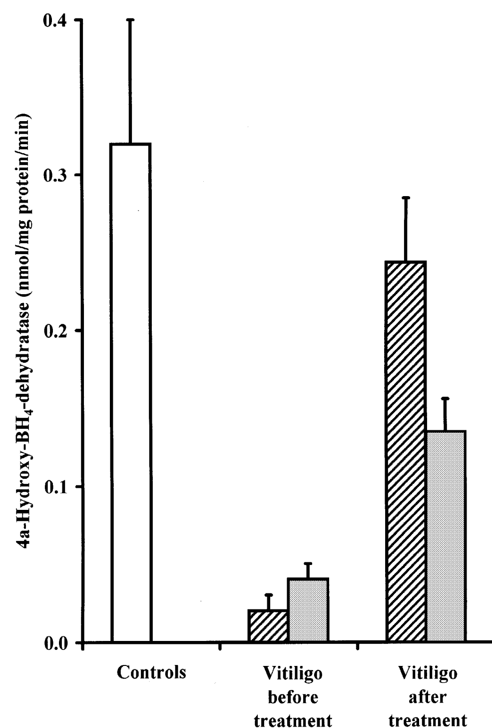

Figure 4. DH enzyme activities in epidermal cell extracts before and after treatment with pseudocatalase. Recovery of DH activities after 6 mo treatment with pseudocatalase ($n = 6$) compared with untreated patients ($n = 7$) and healthy controls ($n = 6$) (error bars show standard deviations). These data confirm the restoration of enzyme activities in the $6BH_4$ recycling process in vitiligo after successful removal of H_2O_2 (▨, nonlesional skin; □, lesional skin) ($p < 0.001 \pm$ SEM).

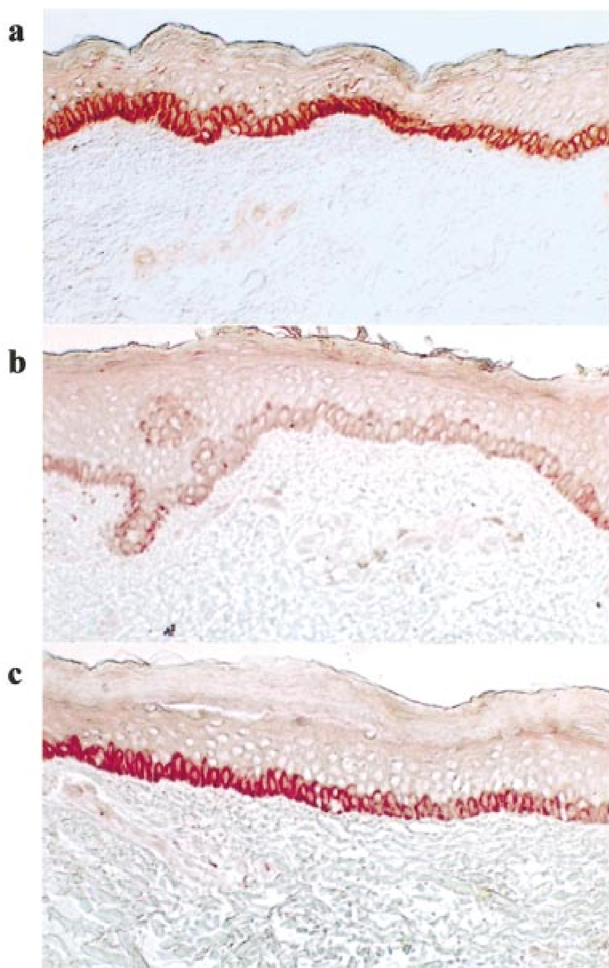

Figure 5. Epidermal DH expression before and after treatment with pseudocatalase. Immunohistochemical detection of DH with the primary monoclonal antibody F 3862 in human skin of (*a*) a healthy control (photo skin type III) and a patient with vitiligo (*b*) before and (*c*) after 6 mo treatment with a UVB-activated pseudocatalase. Note the thickening of the stratum corneum in (*c*) after treatment. DH is predominantly localized in the basal layer, whereas upon differentiation of keratinocytes there is a significantly lower expression of the protein. The section of untreated vitiligo skin shows significantly lower expression in the basal layer, whereas after treatment with a pseudocatalase the expression returns back to normal. As the protein expression recovers after removal of H_2O_2, this result suggested that H_2O_2 affected protein degradation of DH.b

Inactivation of recombinant DH enzyme by H_2O_2 To further substantiate this finding, we incubated purified recombinant DH with H_2O_2 to follow its direct effect on the enzyme activity. We utilized a coupled assay for this purpose as DH can stimulate PAH activity (Kaufman, 1970; Huang *et al*, 1973; Ayling *et al*, 1997). **Figure 7** demonstrates DH-stimulated PAH activity with a 7-fold increase in the initial rate (V_0). This result is in agreement with Kaufman's earlier report (Kaufman, 1970). After preincubation of DH with H_2O_2 for 10 min prior to the PAH assay, however, there was a time-dependent significant decrease of enzyme activity, confirming a H_2O_2-induced deactivation (**Fig 7**).

DH active site alteration by H_2O_2 Molecular modeling of the natural substrate 4a-OH-BH$_4$ in the active site of DH supports previous observations that His[79] and His[61] are responsible for the catalytic activity of the S- and R-isomers of the substrate (Ayling *et al*, 1997). Oxidation of the Cys[81] by H_2O_2, however, affects the orientation of both of these histidine residues, implicating the loss of the hydrogen bond from the imidazole ring of His[79] to the hydroxyl group of the S-isomer in position 4a on the pterin molecule. By contrast, the hydrogen bond from His[61] to the hydroxyl group of the R-isomer remains unaffected. Oxidation of Trp[24] substantially moves both histidine residues and breaks all possible hydrogen bonds with the pterin, resulting in an inactive binding site for the substrate (**Fig 8*a*, *b***). To our knowledge, this observation identifies for the first time the direct deactivation of DH by H_2O_2. Our results exclude the earlier proposed involvement of Trp[65] (Ficner *et al*, 1995).

DH is not mutated in vitiligo Originally low epidermal DH activities implicated the possibility of a leaky mutation in the DH gene (Schallreuter *et al*, 1994a). Therefore, the direct effect of H_2O_2 on the enzyme described above was further supported by an investigation of the DH gene to rule out possible point mutations or mosaics. In all cases studied, we found only wild-type sequences for the DH coding region (data not shown). Based on these results we conclude that low DH activities in vitiligo are not caused by a mutant DH.

DISCUSSION

Over the past the importance of 6BH$_4$ homeostasis in health and disease has been the subject of numerous reports (Smith, 1974; Blau *et al*, 1996; Kaufman, 1997). Previous studies established the presence of a full autocrine 6BH$_4$ synthesis/recycling/regulation in keratinocytes and melanocytes of the human epidermis (Schallreuter *et al*, 1994a; 1994b). Patients with the depigmentation disorder vitiligo exhibit a severely impaired epidermal 6BH$_4$ homeostasis together with an accumulation of H_2O_2 in this compartment (Schallreuter *et al*, 1994a, b, 1999). So far it has remained elusive whether the recycling of 6BH$_4$ (**Fig 1**) with low DH activities in association with high levels of the abiogenic 7BH$_4$ could be caused by a possible mutation of the DH gene or by deactivation of the enzyme itself in these patients (Schallreuter *et al*, 1994a, b). The identification of accumulated 7BH$_4$ is a rather rare event in pterin metabolism (Curtius *et al*, 1988, 1990; Davis *et al*, 1991; Adler *et al*, 1992). To date, it still remains obscure whether this 7-isomer has any physiologic function. 7BH$_4$ accumulation has only been described so far in two disorders, in mild variants of phenylketonuria (PKU) (Curtius *et al*, 1988; Dhondt *et al*, 1988; Citron *et al*, 1993; Ayling *et al*, 2000) and in vitiligo (Schallreuter *et al*, 1994a, b). There is one fundamental difference. Patients affected by the former PKU variants express a transient mild hyperphenylalanaemia and mutations in the DH gene (e.g., from T $\rightarrow$ C causing a substitution of Cys[81] by Arg[81]). Patients with vitiligo show no hyperphenylalanaemia ($n = 321$) (Schallreuter *et al*, 1998; Schallreuter, unpublished results). The results of this study clearly rule out a mutant DH in vitiligo. The removal of epidermal H_2O_2 in patients with vitiligo yields a recovery of DH activities and normal levels of 7BH$_4$ (**Figs 3, 4**). This observation strongly suggests direct involvement of H_2O_2 on this enzyme activity. The immunohistochemical approach in this investigation confirmed the presence of DH in the epidermis of healthy controls and untreated/treated patients, predominantly in the basal layer. Our data support restoration of the protein after removal of H_2O_2 by pseudocatalase (**Fig 5**). In addition, we were able to prove direct deactivation of the enzyme by H_2O_2 with recombinant DH and PAH (**Fig 7**). In this context, it is noteworthy that the enzyme DH functions as a tetramer of identical subunits, whereas its dimers serve for the dimerization of the transcription factor hepatocyte nuclear factor 1 (Ficner *et al*, 1995; Kim and Burley, 1995). Studies employing X-ray crystallography elucidated a binding site for the substrate analog 7,8 dihydrobiopterin on the enzyme (Ficner *et al*, 1995). From these studies, it was proposed that the analog/substrate would bind between the

subunits of the tetramer. Accordingly, Cys^{81} and Trp^{65} were implicated in the vicinity of the catalytic histidine residues in the binding site (Ficner *et al*, 1995). As both amino acids would be susceptible to oxidation via H_2O_2, we suspected that this mechanism could account for low DH activities as observed in vitiligo. Therefore, we examined the entire amino acid sequence of DH, which offers only four possibilities for H_2O_2 oxidation, i.e., Trp^{24}, Trp^{65}, Cys^{81}, Met^{102}. Computer modeling using the natural substrate 4a-OH-BH_4 clearly identifies the oxidation of Cys^{81}, which severely affects the structure of the active site for the binding of the S-isomer, whereas the oxidation of Cys^{81} in the binding of the R-isomer does not alter the enzyme. However, the oxidation of Trp^{24} changes the binding site for both isomers (**Fig 8*a*, *b***). By contrast, Trp^{65} and the methionine residue are located in hydrophobic areas of the protein and therefore do not affect the overall structure of the enzyme.

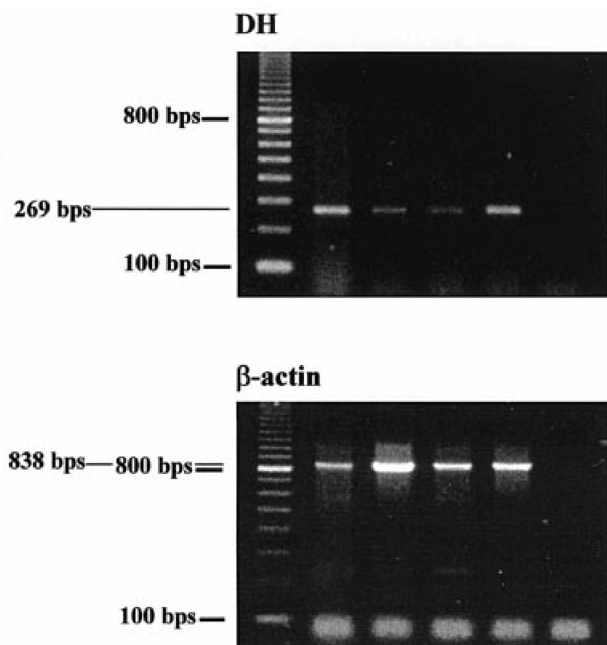

Figure 6. DH *in vitro* mRNA expression in human epidermal melanocytes and keratinocytes from normal healthy and vitiliginous probands. Qualitative agarose gel electrophoretic analysis of PCR assays for DH mRNA expression, standardized to β-actin. The order of the samples on the gel is, from left to right, 100 bp ladder, normal healthy keratinocyte, normal healthy melanocyte, lesional vitiliginous melanocyte, lesional vitiliginous keratinocyte, negative control (omission of template cDNA). This lower expression of melanocyte mRNA can be explained by the observation that melanins can inhibit the PCR (Eckhart *et al*, 2000).

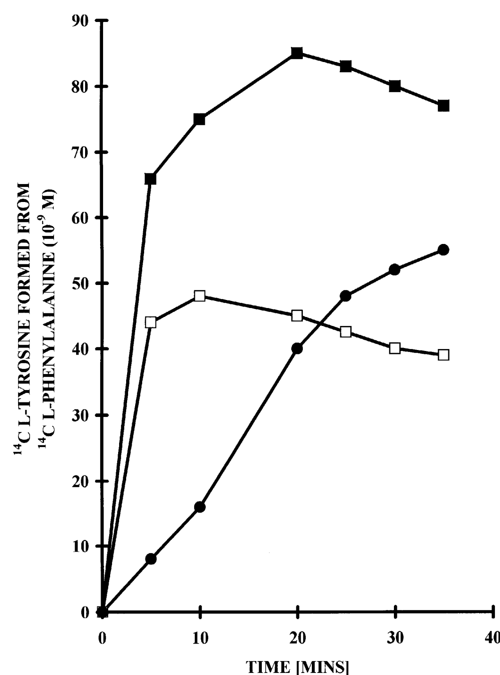

Figure 7. The effect of H_2O_2 on the activation of PAH by DH. The production of ^{14}C L-tyrosine from ^{14}C L-phenylalanine was followed after 35 min by PAH activity alone (●). The initial rate (V_0) of PAH activity was stimulated 7-fold by the addition of recombinant DH (1:1) (■). In order to examine the direct influences of H_2O_2 on DH coupling to PAH, rDH was preincubated with 10 μM of H_2O_2 and the activation assay was repeated showing a time-dependent deactivation of this coupled system (□). Note that, at the incubation time of 10 min, the initial rate of DH activity (V_0) is only reduced by 40%. After 10 min, however, the active coupling is terminated, proving the total loss of DH activity by H_2O_2.

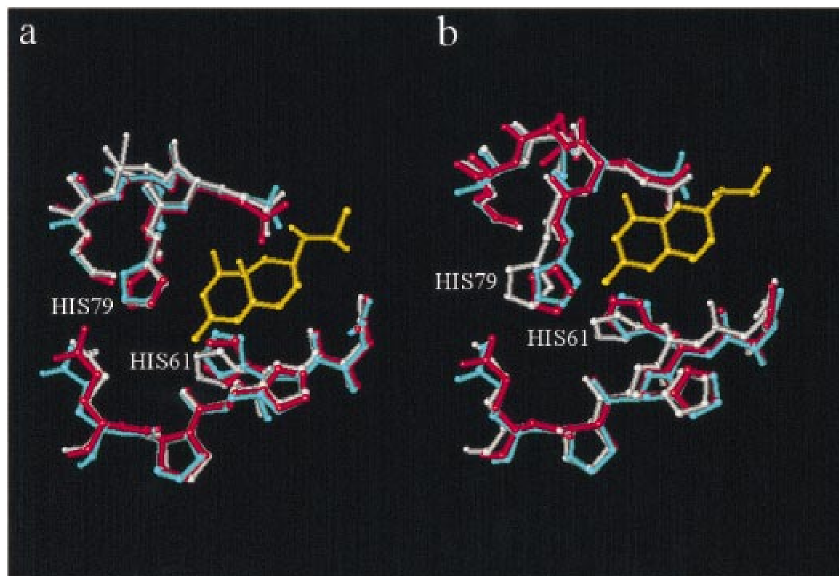

Figure 8. Molecular modeling of the DH active site before and after oxidation with H_2O_2. (*a*) Binding of the pterin S-isomer. (*b*) Binding of the pterin R-isomer. The substrate 4a-OH-BH_4 is shown in yellow. The backbone of the active site of the enzyme is demonstrated in green. The effect of the oxidation of the Cys^{81} residue in the enzyme is presented in red and shows a conformational change only for the S-isomer (*a*) whereas the R-isomer does not affect the enzyme structure (*b*). The reaction of H_2O_2 with Trp^{24} changes the binding of both isomers (white backbones). Oxidation of this residue by H_2O_2 results in the movement of the active site imidazole rings of His^{61} and His^{79}.

To our knowledge, this paper identifies for the first time direct deactivation of the major 6BH$_4$ recycling enzyme, DH, by H_2O_2, both *in vivo* by utilizing the biologic model of the depigmentation disorder vitiligo and *in vitro* by employing recombinant enzymes. In addition, as 6BH$_4$ is an extremely ubiquitous cofactor involved in several important metabolic pathways, our observation could indicate a general mechanism for regulation of the 6BH$_4$ recycling process via H_2O_2. In this context, it has recently been shown that H_2O_2 induces activation of dendritic cells followed by selective T cell proliferation (Rutault *et al*, 1999). As both cell types hold the capacity for 6BH$_4$ synthesis and recycling, our results presented in this study could suggest an H_2O_2/6BH$_4$-mediated control of the immune response. Current studies in our laboratory are under way to further elucidate this interesting hypothesis. Furthermore, it would be of great interest to follow the influence of H_2O_2 on the *de novo* synthesis of 6BH$_4$ in vitiligo.

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