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Increased monoamine oxidase A activity in the epidermis of patients with vitiligo

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Abstract **Human keratinocytes under in vitro conditions synthesize norepinephrine and epinephrine, whereas melanocytes lack this capacity. Keratinocytes established from lesional and nonlesional skin of patients with vitiligo synthesized four and two times more norepinephrine, respectively, than controls. Epinephrine synthesis was similar in keratinocytes from uninvolved epidermis and controls, but cells from involved skin had 6.5-fold less epinephrine than controls, indicative of low phenylehtanolamine-*N*-methyl transferase (PNMT) activity. Similar results were obtained in five patients with vitiligo who showed low epinephrine levels in involved epidermis. Both human keratinocytes and melanocytes expressed significant levels of monoamine oxidase A (MAO-A) activities as shown using ^{14}C-labelled 5-hydroxytryptamine as substrate and immunohistochemical staining with mouse monoclonal antibody. MAO-A activities in the total epidermis of patients with vitiligo were increased five- to ten-fold compared with skin of type-matched controls. Similar increases in MAO-A activities were also found in both keratinocytes and melanocytes established in vitro from vitiliginous epidermis. Based on these results, it can be concluded that defective catecholamine synthesis in the epidermis of patients with vitiligo leads to increased levels of norepinephrine with a concomitant increase in MAO-A activity.**

Key words Monoamine oxidase A · Catecholamines · Vitiligo

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Introduction

Lewinsohn et al. were the first to show that human full-thickness skin contains significant monoamine oxidase A (MAO-A) activity with increasing specific activities from the developing fetus to the adult (i.e. fetal 7, neonatal 179 and adult 326 nmol/mg protein per 30 min) [8]. The contribution of MAO-A activity from the dermis compared with the epidermis was not determined in the experiments of Lewinsohn et al. The recent discovery that human keratinocytes under in vitro conditions and epidermal suction blister extracts have the capacity for catecholamine biosynthesis indicates that the catecholamine degrading enzymes should also be present in the epidermis [13]. Catechol-*o*-methyl-transferase (COMT) has already been found in human keratinocytes, melanocytes and epidermal extracts [6, 7]. However, human melanocytes do not hold the capacity for catecholamine synthesis [13, 15]. To our knowledge, MAO-A activity has not yet been studied in the human epidermis.

Previously, it has been shown that in the depigmentation disorder, vitiligo, there is defective catecholamine biosynthesis with an overproduction of norepinephrine (27/28 patients had a mean plasma content of 575 ng/l, range 377–893 ng/l, compared with controls with < 300 ng/l) [15]. Urine also yields increased norepinephrine and its MAO-A-derived metabolites in patients with active disease [10, 15]. Furthermore, increased plasma norepinephrine levels have been reported [3]. In addition, COMT activity and COMT metabolites are increased in the lesional epidermis of vitiligo patients [7, 10]. Recent studies have shown that, in patients with active vitiligo, the catecholamine biosynthetic pathway is stimulated by an increased synthesis of the essential cofactor (6*R*)-5,6,7,8-tetrahydrobiopterin (6-BH_4) [14, 15] which is the rate-limiting cofactor/electron donor for tyrosine hydroxylase (TH), the key enzyme in the catecholamine pathway, producing L-dopa from L-tyrosine [15]. The increased *de novo* synthesis of 6-BH_4 appears to be due to defective recycling of this cofactor by 4a-hydroxy-6-BH_4 dehydratase

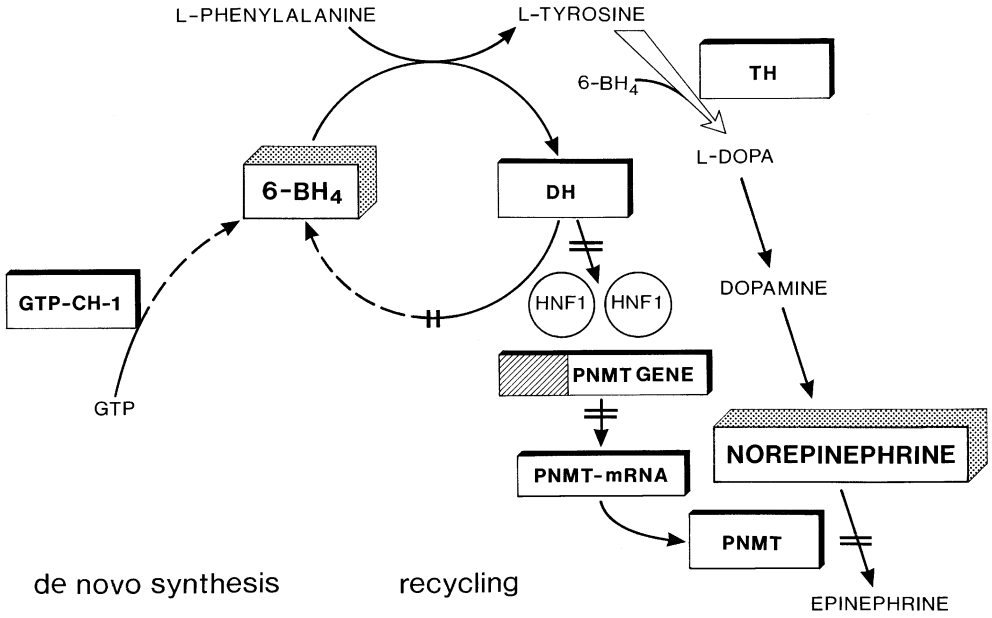

Fig. 1 A proposed mechanism for the accumulation of norepinephrine in the depigmented epidermis of patients with vitiligo. The *de novo* synthesis of 6-BH_4 is controlled by GTP-cyclohydrolase-1 (GTP-CH-1). Once formed, 6-BH_4 is recycled via the rate-limiting enzyme 4a-hydroxy-6-BH_4 dehydratase (DH). DH also controls the transcription of the PNMT gene via the transcription factor hepatocyte nuclear factor 1 (HNF-1). In vitiligo, DH is defective, resulting in increased *de novo* synthesis of 6-BH_4 [12, 13] leading to stimulation of the catecholamine pathway via the activation of tyrosine hydroxylase (TH); hence the downregulation of PNMT due to defective transcription of the PNMT gene causes increased norepinephrine

(DH) [14, 15]. Together with the transcription factor, hepatocyte nuclear factor 1 (HNF-1), DH can act as a dimerization catalyst to regulate the transcription of the phenylethanolamine-*N*-methyl transferase (PNMT) gene by reacting with a 16-base binding sequence on its promoter with control of transcription [15]. In vitiligo, the lesional epidermis expresses significnatly lower than normal PNMT activity in all patients [15]. The increased activity of TH, together with decreased PNMT activities could be a rationale for the observed increase in norepinephrine and its metabolites [15]. A proposed mechanism for increased biosynthesis of norepinephrine in the lesional skin of vitiligo patients is summarized in Fig. 1. The aim of this study was the examination of epidermal catecholamines and their possible degradation by MAO-A in patients with vitiligo.

Materials and methods

Cell cultures

Human keratinocytes were cultured from epidermal suctionblister material in MCDB-153 medium without the addition of fetal calf serum (n = 6, two controls, two vitiligo/involved and uninvolved). Differentiation was induced by increasing the medium calcium concentration from 0.1×10^{-3} *M* to 1.5×10^{-3} *M* together with the removal of growth factors. Differentiating cells were established by the maintenance of a 0.1×10^{-3} *M* calcium concentration where growth factors were removed [17]. Human melanocytes (n = 5, i.e. three controls and one vitiligo/involved and uninvolved) were established in MCDB-153 medium containing initially 10% fetal calf serum and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). TPA was removed and the serum content of the medium was reduced to 0.2% 7 days prior to enzyme determination [11].

Epidermal cell extracts

Epidermal suction blister roofs were obtained from ten vitiligo patients and eight healthy human volunteers using a suction blister device as described elsewhere [5]. Cell extracts were prepared by ultrasonication.

Monoamine oxidase A assay

MAO-A activity was determined by the radiometric microassay described by Lewinsohn et al. [8]. The reaction mixtures contained 170 µmol [^{14}C] hydroxytryptamine and 20 µl cell extract in 0.1 *M* KH_2PO_4 buffer, pH 7.2. The total volume was 235 µl. The reaction mixtures were incubated at 37° C and the reaction stopped at different times by the addition of 100 µl 2 *M* citric acid. The ^{14}C-aldehyde reaction product was extracted into toluene/ethyl acetate (1 : 1 v/v) and 1.0 ml of the organic layer was counted in 3.0 ml aqueous/nonaqueous scintillation fluid (Instant Gel, F.A. Packard, A. Canberra Company) on the ^{14}C-channel. Specific activities for MAO-A were determined from the initial reaction rates as nanomoles aldehyde formed per milligram protein per minute. Protein concentrations were determined by the method of Kalb and Bernlohr [4].

Immunohistochemical staining of MAO-A with monoclonal antibody

The presence of MAO-A was shown using immunoperoxidase staining and a mouse monoclonal antibody to MAO-A.

Determination of catecholamines in cell extracts

Catecholamines were determined using the HPLC method described by Weicker et al. [16].

Materials

[^{14}C]Hydroxytryptamine (specific activity 55 mCi/mmol) was purchased from Amersham Buchler, Braunschweig, Germany. MAO-A mouse monoclonal antibody was obtained from Sertec. All other reagents were from Sigma Chemical Company, St. Louis, Mo., USA.

Results

Norepinephrine and epinephrine were determined in cell extracts from keratinocytes of two patients with vitiligo and compared with the levels in extracts from keratinocytes from two healthy controls under the same experimental conditions. In addition, both catecholamines were measured in epidermal cell extracts from suction blisters of five patients with vitiligo and compared with suction blister extracts from three controls. The results are summarized in Tables 1 and 2. Undifferentiated keratinocytes established from uninvolved skin of two patients with vitiligo showed a fourfold higher norepinephrine content, whereas the epinephrine level was not significantly different compared with control cells. However, the undifferentiated keratinocytes from involved epidermis showed only a twofold increase in norepinephrine and epinephrine was 6.5-fold lower compared with controls (Table 1).

Table 1 Catecholamine concentrations (± SD) in cell extracts from undifferentiated keratinocytes

	Norepinephrine (fmol/mg protein)	Epinephrine (fmol/mg protein)
Control (n = 2)	73.2 (± 10.2)	267.9 (± 35.8)
Vitiligo (uninvolved) (n = 2)	305.5 (± 42.3)	321.7 (± 47.3)
Vitiligo (involved) (n = 2)	157.5 (± 26.4)	41.7 (± 6.2)

Table 2 Catecholamine concentrations (± SD) in cell extracts from epidermal suction blisters

	Norepinephrine (fmol/mg protein)	Epinephrine (fmol/mg protein)
Control (n = 3)	19.6 (± 4.2)	18.1 (± 3.2)
Vitiligo (uninvolved) (n = 5)	44.2 (± 6.0)	36.3 (± 3.9)
Vitiligo (involved) (n = 5)	58.9 (± 4.3)	24.6 (± 4.0)

The results from cell extracts from epidermal blister material did not show the striking differences for norepinephrine observed with cell extracts from pure keratinocyte cell cultures. In the involved epidermis epinephrine levels were significantly lower (30%), consistent with decreased PNMT activities reported previously [15]. Total catecholamine levels (norepinephrine plus epinephrine) were identical in involved and uninvolved epidermis, although the ratio of norepinephrine to epinephrine in uninvolved skin was 1.2 compared with 2.4 in involved epidermis. However, control catecholamine levels were significantly lower (Table 2).

Human undifferentiated, differentiating, and differentiated keratinocytes under in vitro conditions express high MAO-A activities similar to those found in liver [8]. The presence of MAO-A has been confirmed in undifferentiated keratinocytes using mouse monoclonal antibody and immunoperoxidase staining (data not shown).

Figure 2a shows the reaction rates for keratinocytes indicating a futher rapid degradation of the aldehyde product to more polar metabolites which could not be extracted into the organic phase under the described experimental conditions. However, this degradation was signifi-

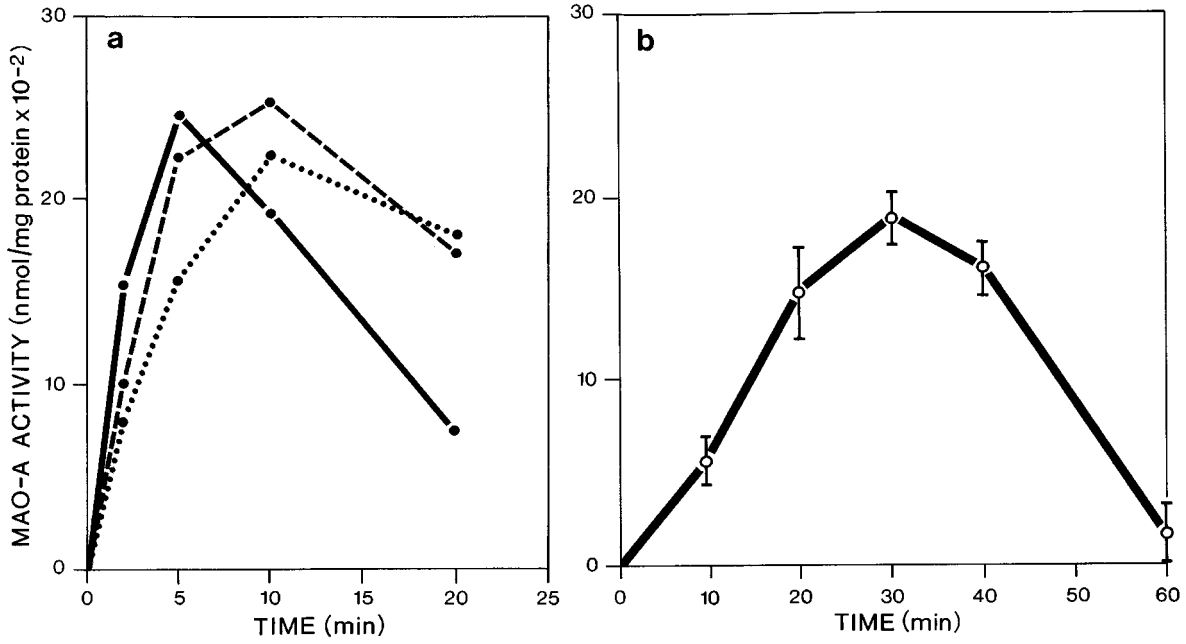

Fig. 2 **a** Kinetics of MAO-A activity in normal keratinocytes (–undifferentiated, … differentiating, - - - differentiated). Further metabolism of the aldehyde product occurs more rapidly in undifferentiated cells. **b** Kinetics of MAO-A activity in epidermal cell extracts from three normal healthy donors measured over 60 min. Degradation of the aldehyde product occurs after 30 minutes

Table 3 MAO-A activities in melanocytes[a]

Melanocytes	MAO-A activity (μmol/mg protein/min)
Control (n = 3)	2.3
Vitiligo (uninvolved) (n = 1)	11.5
Vitiligo (involved) (n = 1)	6.7

[a] To the best of our knowledge, melanocytes from involved epidermis have not been successfully established and passaged before. Therefore, a comparative study on melanocytes of both uninvolved/involved epidermis in duplicate could only be considered for one patient at this time

Table 4 MAO-A activities in keratinocytes

	MAO-A activity (μmol/mg protein/min)
Control (n = 2)	2.7
Vitiligo (uninvolved) (n = 2)	19.5
Vitiligo (involved) (n = 2)	4.1
Control + 200 nmol 6-biopterin (n = 2)	11.0

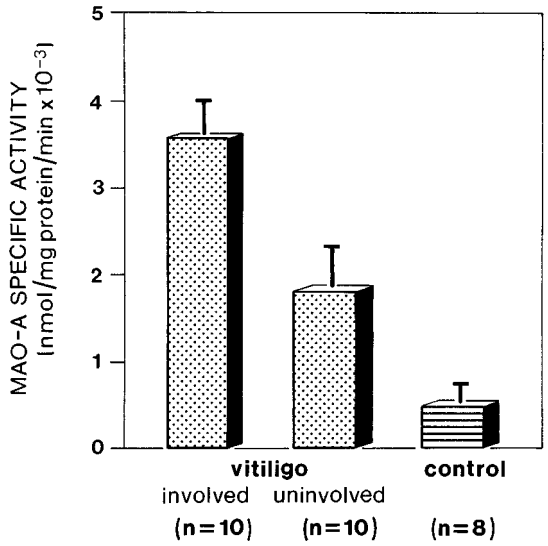

Fig. 3 Mean specific activities for MAO-A in epidermal extracts from ten vitiligo patients from involved and uninvolved epidermis and from eight skin-type-matched controls

cantly more rapid in undifferentiated cells. Figure 2b shows the results from epidermal cell extracts with significantly lower specific MAO-A activities than pure keratinocytes. This decrease may be based on participation of the stratum corneum to protein determination. However, the further rapid metabolism of the aldehyde is comparable to the results from pure keratinocytes.

MAO-A activities determined in keratinocytes and melanocytes from vitiligo patients under in vitro conditions yielded significantly higher enzyme levels in both uninvolved and involved cells compared with controls. The results are summarized in Table 3 and 4, showing a three- to fivefold increase in MAO-A activities in melanocytes and a two- to sevenfold increase in keratinocytes compared with controls.

We have previously been able to show that vitiliginous epidermis (both uninvolved and involved) contains significantly increased levels of biopterins [14, 15]. Therefore, it was of great interest to study the direct influence of 6-biopterin on MAO-A activity. Upon addition of 200 nmol 6-biopterin to control keratinocytes, a fivefold increase in MAO-A activity was observed (Table 4).

Consistent with the results with cells, MAO-A activities in epidermal suction blister extracts from the ten patients with vitiligo were significantly increased compared with age/skin-type-matched controls. The results are presented in Fig. 3.

Discussion

Patients with vitiligo have defective catecholamine biosynthesis with increased norepinephrine levels in their epidermis and in their plasma [10, 15]. Keratinocytes established from vitiliginous skin continue to overproduce norepinephrine even after several passages. Both cells from the depigmented epidermis of these patients and extracts from suction blister roofs contained lower than normal levels of epinephrine indicative of low PNMT activity, as determined previously in the involved epidermis of these patients [15]. A consequence of increased norepinephrine appears to be the induction of the catecholamine-degrading enzymes MAO-A and COMT [6, 7]. It has previously been reported that MAO-A has both constitutive and inducible isoforms where norepinephrine is the preferred substrate and inducer of this enzyme in neuronal tissue [18]. MAO-A was found to oxidize both norepinephrine and epinephrine yielding ammonia, 3,4-dihydroxymandelic aldehyde and hydrogen peroxide (Fig. 4). The aldehyde product was further metabolized to other more polar degradation products. Human keratinocytes during the total differentiation process (undifferentiated, differentiating and differentiated), expressed high levels of MAO-A with a rapid further metabolism as shown in Fig. 2a. The aldehyde produced by MAO-A in keratinocytes and total epidermis must yield more polar, nonsolvent extractable metabolites. However, undifferenti-

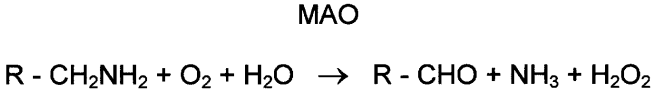

Fig. 4 General reaction scheme for the oxidation of monoamines by MAO with the stoichiometric production of aldehyde, ammonia and hydrogen peroxide

ated keratinocytes were more active in the synthesis and metabolism of the aldehyde produced by MAO-A compared with either differentiating or differentiated cells.

A comparative study of MAO-A in undifferentiated keratinocytes and melanocytes established from one patient with vitiligo revealed significantly increased enzyme activities compared with age/skin-type-matched controls (Tables 3 and 4). Also, the results with cell extracts from epidermal suction blisters, confirmed the results obtained with cells in vitro showing a 10.5-fold increased activity in depigmented epidermis versus a 5.5-fold increased activity in non-lesional skin compared with controls. MAO-A was induced to levels similar to those found in vitiliginous cells when control keratinocytes were cultured in the presence of 200 nm 6-biopterin (Table 4). These results prove that increased 6-BH_4 synthesis in vitiligo is associated with an overproduction of norepinephrine directly stimulating the upregulation of both MAO-A and COMT degradative pathways in these patients [6, 7].

One consequence of increased MAO-A activity in the epidermis of patients with vitiligo is the accumulation of toxic levels of hydrogen peroxide. In addition, the defective recycling of 6-BH_4 in vitiligo leads also to an increased production of hydrogen peroxide [14, 15]. Peroxidative damage to cells in the epidermis of vitiligo patients has been suspected previously with the discovery of low catalase activity, and from histologic observations of membrane damage [2, 9, 12]. Based on these observations, it could be concluded that low catalase activities in the epidermis of patients with vitiligo most likely originate from oxidative damage to the heme active site of this enzyme by a localized burst of hydrogen peroxide as described previously by Aronoff [1].

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