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Human phenylalanine hydroxylase is activated by H_2O_2: a novel mechanism for increasing the L-tyrosine supply for melanogenesis in melanocytes

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Abstract

Epidermal phenylalanine hydroxylase (PAH) produces L-tyrosine from the essential amino acid L-phenylalanine supporting melanogenesis in human melanocytes. Those PAH activities increase linearly in the different skin phototypes I–VI (Fitzpatrick classification) and also increase up to 24 h after UVB light with only one minimal erythemal dose. Since UVB generates also H_2O_2, we here asked the question whether this reactive oxygen species could influence the activity of pure recombinant human PAH. Under saturating conditions with the substrate L-phenylalanine (1×10^{-3} M), the V_{max} for enzyme activity increased 4-fold by H_2O_2 ($>2.0 \times 10^{-3}$ M). Lineweaver–Burk analysis identified a mixed activation mechanism involving both the regulatory and catalytic domains of PAH. Hyperchem molecular modelling and Deep View analysis support oxidation of the single Trp^{120} residue to 5-OH-Trp^{120} by H_2O_2 causing a conformational change in the regulatory domain. PAH was still activated by H_2O_2 in the presence of the electron donor/cofactor 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin despite slow oxidation of this cofactor. In vivo FT-Raman spectroscopy confirmed decreased epidermal phenylalanine in association with increased tyrosine after UVB exposure. Hence, generation of H_2O_2 by UVB can activate epidermal PAH leading to an increased L-tyrosine pool for melanogenesis.

Keywords: Phenylalanine hydroxylase; Phenylalanine; Tyrosine; H_2O_2; UVB; Tetrahydrobiopterin; Melanogenesis; Human epidermis

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) is the rate limiting enzyme for the irreversible oxidation of the essential amino acid L-phenylalanine to L-tyrosine [1]. PAH belongs to a class of aromatic amino acid hydroxylases, including tyrosine hydroxylase (TH, EC 1.14.16.2) and tryptophan hydroxylase (TrpOH, EC 1.14.16.4), possessing both conserved sequence homology and mechanisms [1]. L-Phenylalanine and O_2 are co-substrates for PAH while 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) is the essential cofactor/electron donor [1,2]. Recently the structure of PAH has been elucidated containing three domains: (1) a regulatory domain (residues 1–142), (2) a catalytic domain (residues 143–410), and (3) a tetramerization domain (residues 411–452). Moreover, the active site has a non-heme iron atom, which is essential for enzyme activity. PAH requires the binding of L-phenylalanine to the regulatory domain in order to promote phosphorylation of Ser^{16} followed by a conformational change, which allows the formation of active tetramers from inactive precursor dimers [3–6]. Once the active tetrameric form of

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the enzyme has been produced, L-phenylalanine and $6BH_4$ can bind to the active site domain initiating catalysis. In addition, PAH is tightly coupled to pterin-4a-carbinolamine dehydratase (PCD, EC 4.2.1.96), the key enzyme for the recycling of the cofactor $6BH_4$ [7,8]. It has been shown that PCD increases the rate of PAH 7-fold and converts the product 4a-OH-carbinolamine to quinonoid dihydrobiopterin (qBH_2) by dehydration which is further reduced to $6BH_4$ in the presence of NADH by dihydropteridine reductase (DHPR, EC 1.6.99.7) [7–9].

Earlier it has been shown that human epidermal melanocytes and keratinocytes hold the full capacity for $6BH_4$ de novo synthesis/recycling/regulation including PAH enzyme activities [10,11]. These enzyme activities increase linearly with skin phototype I–VI (Fitzpatrick classification) [12,13]. They also increase after exposure to only one minimal erythemal dose (MED) of UVB [14]. In this context, it was demonstrated that melanocytes actively transport L-phenylalanine via a neutral amino acid antiporter coupled to Na^+/Ca^{2+} ATP-ase and these cells turn over this amino acid to L-tyrosine in the cytosol [15]. A comparative study of [^{14}C]L-phenylalanine (100 μM) uptake and turnover compared to [^{3}H]L-tyrosine (100 μM) uptake showed that 65% of the eumelanin produced in melanocytes originate from [^{14}C]L-phenylalanine and only 35% from [^{3}H]L-tyrosine [15]. This result highlighted for the first time the importance of autocrine L-tyrosine synthesis via PAH in melanocytes for melanogenesis.

Since UVB light produces H_2O_2 in the 10^{-3} M range in the human epidermis and UVB increases PAH activities in this compartment, we were interested to explore whether H_2O_2 influences the activity of pure recombinant human PAH [14–16]. The results presented herein show for the first time that H_2O_2 increases the V_{max} of PAH 4-fold. Hence, UVB generated H_2O_2 upregulates the supply of L-tyrosine in the human epidermis promoting de novo melanogenesis. Further support stems from in vivo FT-Raman spectroscopy by following the phenylalanine/tyrosine turnover before and after UVB exposure. These results demonstrate a decrease of the phenylalanine signal at 1004 cm^{-1} concomitant with the rise of the tyrosine peak at 846 cm^{-1}.

Materials and methods

6(*R*)-L-Erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) was obtained from Schircks Laboratories (Jona, Switzerland). All other reagents and chemicals were from Sigma (Poole/Dorset, UK). Recombinant human PAH was produced in the Department of Biochemistry and Molecular Biology, University of Bergen, and was a generous gift from Professor Aurora Martinez.

PAH enzyme assays. PAH activities were followed by measuring the formation of L-tyrosine from L-phenylalanine at 278 nm in Hepes buffer 0.02 M with 0.2 M NaCl at pH 7.05. Pre-activation of PAH by L-phenylalanine for 2 min was required prior to the addition of the cofactor/electron donor $6BH_4$. Reactions were linear and reproducible over 6 min. Reaction rates were determined between 3 and 6 min by $\Delta OD_{278\,nm}$/3 min. In order to determine the optimum concentration of H_2O_2 for full activation, reactions with H_2O_2 were measured with saturating levels of L-phenylalanine (1×10^{-3} M) and $6BH_4$ (240×10^{-6} M). The mechanism of the H_2O_2 interaction was followed utilising V vs S analysis and Lineweaver–Burk activation plots in the presence of 2.0×10^{-3} M H_2O_2.

In vivo detection of epidermal phenylalanine/tyrosine by FT-Raman spectroscopy. FT-Raman spectroscopy was performed with a BRUKER RFS 100/S spectrometer equipped with liquid nitrogen cooled germanium detector and a fibre optic cable. Sample excitation was accomplished using a Nd^{3+}/YAG laser operating at 1064 nm with a laser power of 200 mW. Each spectrum was accumulated in 2 min with 200 scans and resolution of 4 cm^{-1}. The spectra were normalised using the CH_2 scissoring vibration located at 1442 cm^{-1}. Total phenylalanine was visualised as a well-resolved peak at 1004 cm^{-1} based on the breathing vibration of the aromatic ring [17,18]. The total tyrosine was visualised at 846 cm^{-1}.

Spectra were taken on sun unexposed skin of the inner forearm (skin phototype III, Fitzpatrick classification) of healthy human volunteers before and 5 min after UVB exposure with one minimal erythemal dose (MED). This study was approved by the Local Ethics Committee.

Hyperchem computer modelling. A structural model of the regulatory domains of native and H_2O_2-oxidised human PAH was created and minimised using Deep View analysis and Hyperchem software. The model was based on the published X-ray crystal structure on the enzyme [3]. Structures were compared after oxidation of the single Trp^{120} residue to 5-OH-Trp^{120} in both the native and oxidised enzyme (residues 1–142).

Results

Recombinant PAH is activated by H_2O_2

Rates for PAH activity were determined in the presence of $0-5 \times 10^{-3}$ M H_2O_2. Optimal activation occurred at concentrations $>2.0 \times 10^{-3}$ M (Fig. 1). First a kinetic analysis of the activation of PAH by 2.0×10^{-3} M H_2O_2 was performed in the presence of

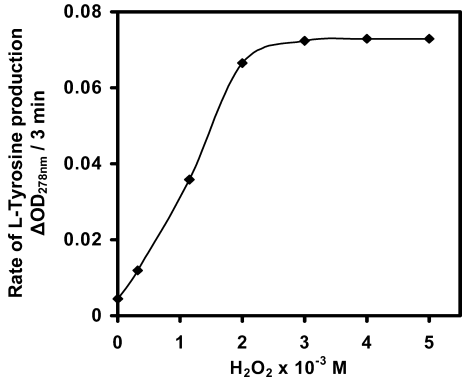

Fig. 1. Concentration dependent activation of PAH by H_2O_2. PAH is activated by H_2O_2 ($0–5 \times 10^{-3}$ M) in the presence of saturating L-phenylalanine (1.0×10^{-3} M) and $6BH_4$ (2.4×10^{-4} M). Reactions contained 0.02 M Hepes buffer with 0.2 M NaCl, pH 7.05, 30 μg rhPAH and were started with $6BH_4$.

different concentrations of the substrate/activator L-phenylalanine. V vs S and Lineweaver–Burk analysis of these results showed a 4-fold increase in V_{max} and a decrease in K_m from 40 to 28×10^{-6} M with 2.0×10^{-3} M H_2O_2. The Lineweaver–Burk plot indicated mixed activation suggesting that H_2O_2 influenced both the regulatory and the active site domains (Figs. 2A and B). Since the binding of the cofactor/electron donor $6BH_4$ requires binding of L-phenylalanine to both its regulatory and active sites, a kinetic analysis of PAH activities in the presence of 2.0×10^{-3} M H_2O_2 and different concentrations of $6BH_4$ was performed. V vs S plots with increasing concentrations of cofactor confirmed activation of PAH (Fig. 3). The result in Fig. 3 is not as precise as with L-phenylalanine alone (Fig. 2A) because $6BH_4$ is slowly oxidised by H_2O_2 to 7,8-dihydrobiopterin and finally to 6-biopterin [19,20]. Both pterins are competitive inhibitors of $6BH_4$ but cannot promote catalysis [1,2]. The results show that H_2O_2 facilitates the rapid activation of PAH despite the slow concentration dependent oxidation of $6BH_4$ (Fig. 3).

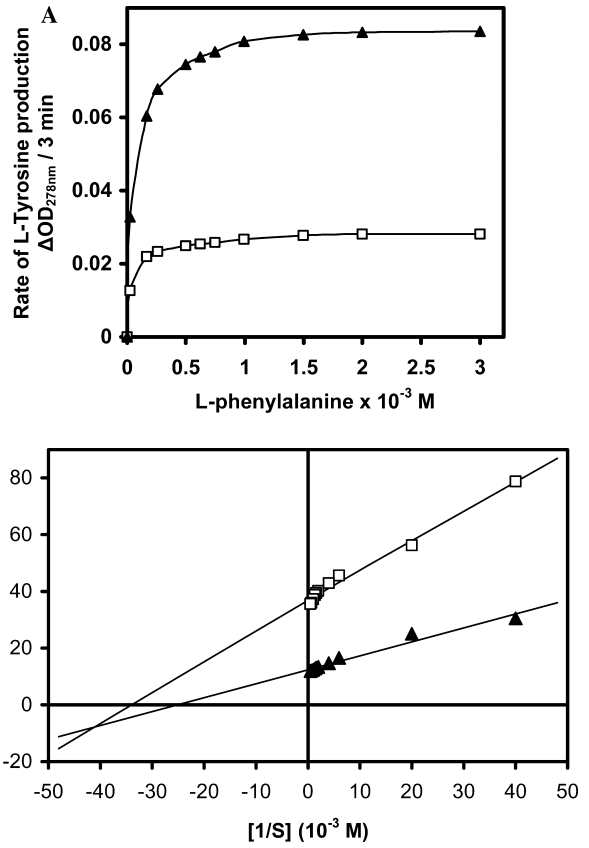

Fig. 2. PAH activation by H_2O_2 (2.0×10^{-3} M) in the presence of the substrate L-phenylalanine. (A) V vs S plots of activated PAH (▲–▲) compared to the control with L-phenylalanine (0–3.25×10^{-3} M) alone (□–□) shows a 4-fold increased V_{max} after H_2O_2. (B) Lineweaver–Burk plots of H_2O_2 activated PAH These data show mixed kinetics for activation and confirm the 4-fold increase in V_{max} and indicate that both regulatory and catalytic domains of PAH are affected by H_2O_2. [H_2O_2 activated PAH (▲–▲), control (□–□).]

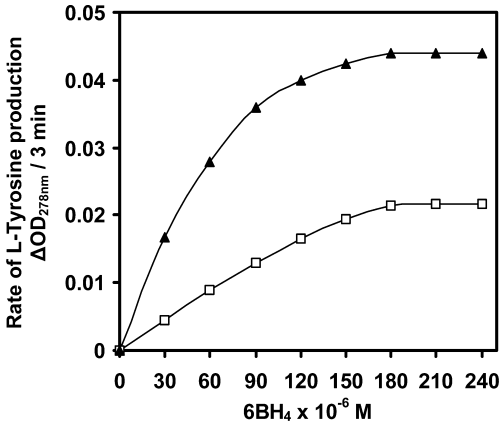

Fig. 3. V vs S plots of PAH activation in the presence of 2.0×10^{-3} M H_2O_2 and the cofactor $6BH_4$ (0–2.4×10^{-4} M). The activation of PAH is only 2.5-fold, because $6BH_4$ is slowly oxidised by H_2O_2 [19,20]. [H_2O_2 activated PAH (▲–▲), control (□–□).]

Molecular modelling of the regulatory domain (residues 1–142) in the presence and absence of H_2O_2

The regulatory domain of human recombinant PAH has only one Trp^{120} residue susceptible to direct oxidation by H_2O_2. It is positioned at the end of a short β-pleated sheet close to a short α-helix at residues 126–129 in the sequence [3,6] (Fig. 4A). Oxidation of Trp^{120} causes a significant change in the spatial orientation of this residue leading to several effects on the tertiary structure of the molecule. The most pronounced changes are close to the Trp residue itself. After oxidation a pronounced shift of the α-helical region ^{125}QGLD^{129} is observed. There are also other changes including a shift of a β-sheet from residues ^{34}AISLIFSLKE^{43} together with some movement of the N-terminal loops nearby similar to the documented shift after phosphorylation of Ser^{16} [6]. The comparative model suggests a very subtle shifting of two other helices

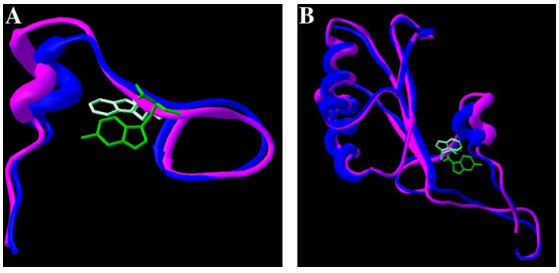

Fig. 4. Hyperchem modelling with Deep View analysis of the regulatory domain of human rPAH before and after H_2O_2 oxidation. (A) Structural changes in the vicinity of Trp^{120} (pale green) after oxidation to 5-OH-Trp^{120} (dark green). N.B. The significant change of the structure of the α-helix close to Trp^{120} at residues 126–129. (B) Deep View analysis of the entire regulatory domain of rPAH (residues 1–142) after oxidation of Trp^{120} yields a significant conformational shift at the N-terminus. This result is similar to activation by phosphorylation of Ser^{16} in the sequence.

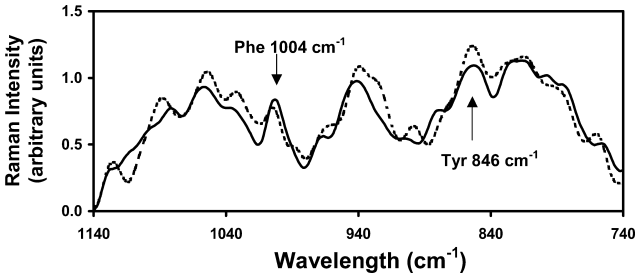

Fig. 5. In vivo FT-Raman spectra (200 scans) of the epidermis before and after UVB exposure (1 MED) N.B. The total phenylalanine is decreased associated with a concomitant increase in total tyrosine after UVB exposure. [Before (——), after (· · · · · ·).]

in the regulatory domain, one from ^{47}ALAKVLRLFE^{56} and the other from ^{92}GSIIKSLRND^{101}. Oxidation of Trp by H_2O_2 also extends this helix involving the residues ^{89}PVL^{92}. There are also changes in several other regions of the loops in the regulatory domain. Taken together these changes could support the 4-fold activation of PAH by H_2O_2 (Figs. 4A and B).

In vivo FT-Raman spectroscopy confirms phenylalanine turnover after UVB (1 MED)

Total epidermal phenylalanine was shown as a well-resolved peak at 1004 cm^{-1} and total tyrosine occurred at 846 cm^{-1}. The spectra after UVB exposure demonstrated a decrease in the total phenylalanine peak concomitant with an increase in the total tyrosine (Fig. 5). This in vivo result is in agreement with H_2O_2 generation in the 10^{-3} M range after UVB exposure [16] and supports the above in vitro results on H_2O_2 mediated PAH activation.

Discussion

Over the past it was shown that H_2O_2 has a dual role in the control of pigmentation in the human epidermis [8,13,14,21]. At low concentrations it activates several important enzymes that control melanogenesis, but at high concentrations it is a powerful inhibitor. H_2O_2 activates tyrosinase (EC 1.14.18.1) significantly at concentrations of 3×10^{-4} M, whereas the enzyme is deactivated in the presence of 10^{-3} M H_2O_2. The same ROS increases the transcription of GTP-cyclohydrolase I (GTP-CH-1, EC 3.5.4.16), the rate limiting enzyme for the de novo synthesis of $6BH_4$ as well as the expression and activities of PCD and DHPR, the enzymes responsible for $6BH_4$ recycling [8,9,21]. In this report we show for the first time that PAH is increased 4-fold by H_2O_2 in the 10^{-3} M range (Figs. 2A, B and 3). Computer modelling of the regulatory domain (residues 1–142) of human recombinant PAH supported oxidation of the single Trp^{120} residue to 5-OH-Trp in the H_2O_2 mediated activation of the enzyme as indicated by the mixed mechanism in the kinetic analysis (Fig. 2B). Direct oxidation of the Fe^{II} active site by H_2O_2 instead of O_2 can explain the competitive component of the kinetics presented in Figs. 2A and B. This activation is significantly higher compared to the phosphorylation of Ser^{16} in the enzyme, which has only a 2.5-fold increase in V_{max} [6]. Moreover, H_2O_2 in the 10^{-3} M range deactivates both PCD and DHPR by oxidising important Met and Trp residues in their active sites [8,9].

Melanogenesis depends on the supply of L-tyrosine and the activity of tyrosinase, the key enzyme in this process. Earlier it has been shown that pigmentation in the human epidermis relies on the autocrine synthesis of L-tyrosine from L-phenylalanine by PAH in the melanocyte [15]. The results presented herein identify a novel additional mechanism to promote de novo melanogenesis because the generation of H_2O_2 by UVB light is sufficient to activate PAH and can explain on one hand the increase of PAH activities as observed after one MED UVB exposure and on the other hand the initiated phenylalanine turnover confirmed by in vivo FT-Raman spectroscopy.

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