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CYTOTOXICITY OF 6-BIOPTERIN TO HUMAN MELANOCYTES

Karin U. Schallreuter[1], Gerd Büttner[1], Mark R. Pittelkow[2], John M. Wood[3], Norma N. Swanson[2], and Christa Körner[1]

[1]Department of Dermatology, University of Hamburg, Hamburg, GERMANY

[2]Department of Dermatology, Mayo Clinic, Rochester, Minnesota

[3]Department of Biomedical Sciences, University of Bradford, Bradford, U.K.

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(6R)5,6,7,8 tetrahydrobiopterin (6-BH_4) is an important cofactor in the regulation of melanogenesis in melanocytes, where it controls: (a) the supply of L-tyrosine from L-phenylalanine via phenylalanine hydroxylase, and (b) regulates directly dopaquinone formation from L-tyrosine via tyrosinase. 6-BH_4 undergoes redox-cycling by its oxidation to quinonoid dihydrobiopterin (qBH_2) and to 6-biopterin through consecutive two electron oxidation reactions. The oxidized cofactor 6-biopterin (0.2 x 10^{-6} M) is extremely cytotoxic to human melanocytes under *in vitro* conditions. Consequently, its reduction to 6-BH_4 via q-BH_2 is essential to melanocyte viability. In addition,, the results herein show for the first time that human thioredoxin reductase has the capacity to reduce 6-biopterin to q-BH_2 where further reduction to 6-BH_4 follows via dihydropteridine reductase or reduced glutathione.

The biosynthesis of the melanins by melanocytes in the human epidermis depends on the essential activity of tyrosinase (monophenol dihydroxyl phenylalanine oxygen oxidoreductase EC 1,14, 8, 1) by catalyzing the oxidation of L-tyrosine to the melanin precursor L-dopaquinone [1]. The activity of tyrosinase requires the supply of its substrates L-tyrosine and superoxide anion radical ($O_2^{\cdot}$) [2-7]. L-tyrosine is synthesized from the essential amino acid L-phenylalanine via L-phenylalanine hydroxylase (L-phenylalanine-tetrahydrobiopterin oxygen oxidoreductase EC 1,14,16,1), where (6R)5,6,7,8 tetrahydrobiopterin (6-BH_4) is the essential cofactor/electron donor for the hydroxylation of L-phenylalanine [8]. Recently, it has been shown that the entire system for *do novo* synthesis and recycling of 6-BH_4 is present in melanocytes [6, 7]. In addition, 6-BH_4

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functions as an uncompetitive inhibitor on tyrosinase directly with a K_I = 13 x 10^{-6} M, but oxidized biopterin does not inhibit this enzyme [9]. As a consequence, it has been expected that the redox cycling of this cofactor directly controls melanogenesis.

MATERIAL AND METHODS

Human melanocytes were established from neonatal foreskin and grown in MCDB 153 medium containing 0.2% FCS, TPA and cholera toxin. Cells used for this study were in the second or third passage. All mitogens were removed five days prior to the experiments and cells were maintained in 0.1% FCS. Human thioredoxin reductase was purified from metastatic melanoma tissue as described previously [10]. 6-BH_4, 7-BH_4, 6-biopterin and 7-biopterin were from Schirks Laboratories, Switzerland. All other reagents were purchased from Sigma/St. Louis, MO.

The viability and morphology of melanocytes was followed microscopically with photographic documentation in the presence of 0-0.2 x 10^{-6} M 6- and 7-biopterin respectively over a time period (0, 6, 12, 24, 48 96 hours). The production of quinonoid dihydropterin (q-BH_2) from 6-biopterin catalyzed by thioredoxin reductase was measured spectophotometrically where q-BH_2 presents a unique spectrum compared to other pterins with a shoulder at 370 nm (E - 3.700 M^{-1} cm^{1}) [5].

Reactions contained 200 µl tris/HCL buffer 0.1 M pH 7.5, 100 µl NADPH (4.0 mg/ml), 200 µl 6-biopterin (1 mg/ml). Reactions were started by the addition of 10 µl of thioredoxin reductase (3.4 mg/ml) in the presence and absence of 10^{-3} M calcium chloride.

RESULTS

Figure 1 presents the microscopic examination of human melanocyte morphology and cell density after a time dependent exposure to 0.2 x 10^{-6} M 6-biopterin. The results show a significant decrease in the cell numbers over time with a concomitant loss of the dendrites and finally, cell death. An IC_{50} of approximately 0.1 x 10^{-6} M was determined after 48 hours exposure to 6-biopterin. By contrast, the abiotic isomer 7-biopterin yielded no cytotoxicity under the same experimental conditions (Figure 2). The mechanism for the two electron reduction of 6-biopterin to a dihydropterin has escaped definition so far. Figure 3 shows that thioredoxin reductase catalyzes a slow reduction of 6-biopterin to q-BH_2 by following the absorption spectrum at 370 nm.

The reduction rate of 6-biopterin to q-BH_2 was inhibited upon addition of 10^{-3} M calcium to the reaction mixture (Figure 3). However, the reduction rate of 6-biopterin was not enhanced

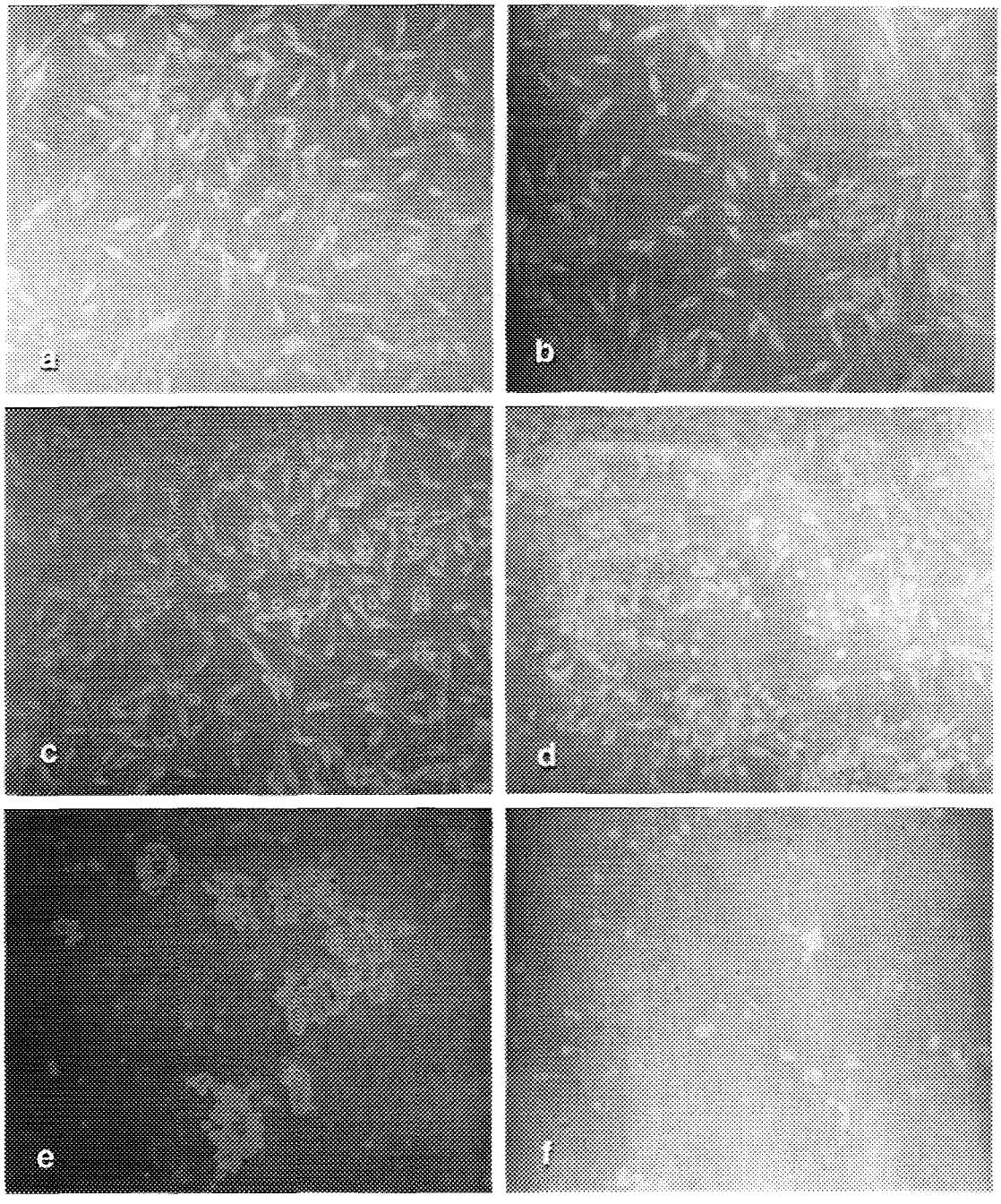

Figure 1.

Microscopic examination of the cytotoxicity of 6-biopterin (0.2×10^{-6} M) to normal human melanocytes (a) control after 6 hours; (b) exposure to 6-biopterin after 6 hours, (c) control after 24 hours, (d) after 24 hours with 6-biopterin, (e) after 48 hours, (f) after 96 hours.

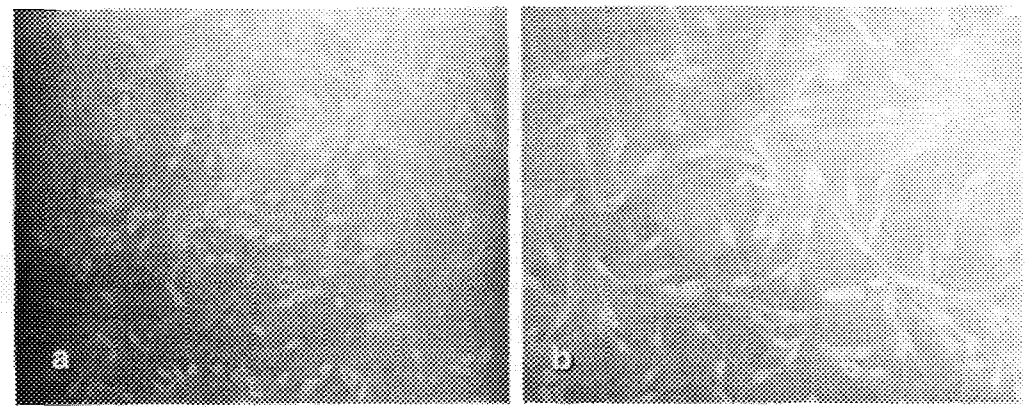

Figure 2.

Microscopic examination of the effect of 7-biopterin (0.2×10^{-6} M) on normal human melanocytes (a) control, (b) after exposure to 7-biopterin (48 hrs.).

after addition of thioredoxin (ADF), indicating a direct reduction of 6-biopterin by the dithiolate active site of thioredoxin reductase.

DISCUSSION

To our knowledge, the data presented herein show for the first time that human melanocytes under *in vitro* conditions yielded a selective cytotoxicity towards 6-biopterin,

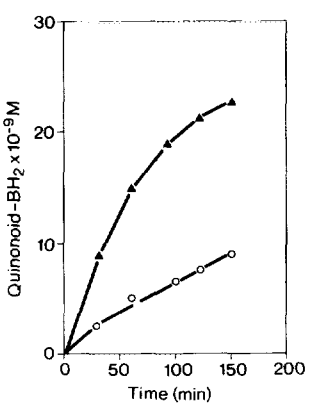

Figure 3.

The reduction of 6-biopterin to quinonoid dihydropterin by human thioredoxin reductase in the presence (○-○) and absence of 10^{-3} M calcium (▲-▲).

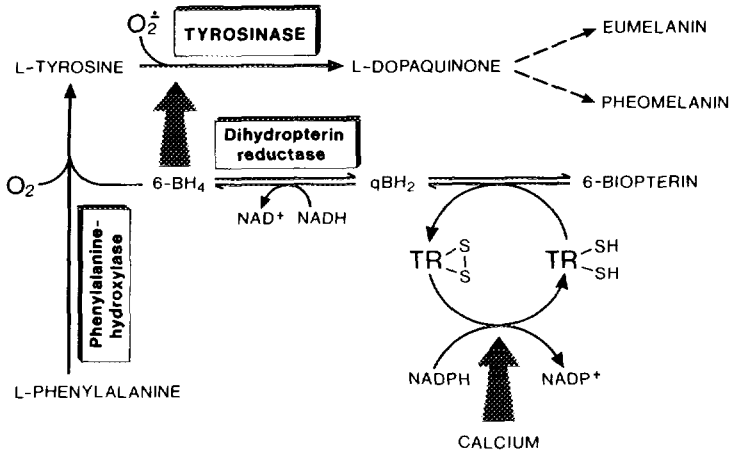

Figure 4.

6-BH_4 is the essential cofactor/electron donor for the production of L-tyrosine from L-phenylalanine via phenylalanine hydroxylase. 6-BH_4 is also an uncompetitive inhibitor of tyrosinase in the conversion of L-tyrosine to L-dopaquinone. L-dopaquinone is the common substrate for the biosynthesis of both eumelanin (black) and pheomelanin (red). Superoxide anion radical ($O_2^{\cdot}$) is generated by UVB light and serves as a substrate for tyrosinase and activates the enzyme by oxidizing 6-BH_4 to 6-biopterin via q-BH_2. The reduction of 6-biopterin to q-BH_2 is catalyzed by thioredoxin reductase (TR) which is inhibited allosterically by calcium. q-BH_2 is reduced to 6-BH_4 either by NADH-dependent dihydropteridine reductase or by reduced glutathione. All of the above enzyme activities including the biosynthesis and recycling of 6-BH_4 have been determined previously in human melanocytes [6, 7].

whereas the abiotic isomer 7-biopterin has no effect on cell morphology and viability. The accumulation of 6-biopterin in the melanocyte could be expected under conditions of oxidative stress where 6-BH_4 will be oxidized to 6-biopterin. Previous results have established that thioredoxin reductase is induced by oxidative stress, whereas superoxide dismutase, catalase and glutathione reductase activities are decreased [3]. Since thioredoxin reductase has the capacity to reduce oxidized 6-biopterin to q-BH_2 which can be further reduced by dihydropterin reductase or reduced glutathione [11], it seems reasonable that this catalyst may play an important role in the redox balance of the epidermis. Also, recently it has been shown that the expression of

thioredoxin reductase in the human epidermis parallels the expression of the constitutive pigment (i.e., skin types I-VI, Fitzpatrick classification) as well as melanogenesis [12, 13]. In addition, it has been shown that thioredoxin reductase is under allosteric control by calcium via a single EF-hands binding site [14]. Hence, it can be concluded that the calcium status of the melanocyte and its external environment will play an important role in a significant reduction of 6-biopterin in order to protect this cell against its cytotoxicity and control melanogenesis. A model for the concerted regulation of melanin biosynthesis is presented in Figure 4.

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