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Activation of melanogenesis by vacuolar type H^+-ATPase inhibitors in amelanotic, tyrosinase positive human and mouse melanoma cells

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Abstract **In this study, we describe the activation of melanogenesis by selective vacuolar type H^+-ATPase inhibitors (bafilomycin A1 and concanamycin A) in amelanotic human and mouse melanoma cells which express tyrosinase but show no melanogenesis. Addition of the inhibitors activated tyrosinase within 4 h, and by 24 h the cells contained measurable amounts of melanin. These effects were not inhibited by cycloheximide (2 μg/ml) which is consistent with a post-translational mechanism of activation. Our findings suggest that melanosomal pH could be an important and dynamic factor in the control of melanogenesis in mammalian cells. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Melanogenesis; Tyrosinase activation; Melanosomal pH

1. Introduction

Melanin production by mammalian pigment cells is a process involving a series of complex cellular events. Despite the recent cloning and characterisation of several enzymes and structural proteins (tyrosinase, tyrosinase-related proteins type 1 and 2 (TRP-1 and -2), silver locus 100 kDa protein (Gp100), melanoma-associated antigen recognised by T cells (MART)) that are involved in the synthesis of melanin [1], our understanding of this process and its regulation is still far from clear. The copper containing metalloenzyme tyrosinase (EC 1.14.18.1) which is specific to melanocytes and catalyses the initial steps in the melanogenesis pathway is considered to be the rate limiting enzyme for melanin synthesis. Its absence, as occurs in the hypopigmentary disorder tyrosinase negative albinism, results in a complete absence of melanin.

As the key determinant for melanin synthesis, tyrosinase is under a sophisticated control by transcriptional and translational mechanisms. Recently, there has been interest in the role of post-translational events in the regulation of tyrosinase activity [2–5]. It is recognised that variations in tyrosinase activity in different pigment cells cannot be explained on the basis of enzyme abundance [6]. For instance, amelanotic and pigmented subclones of melanoma cells can have similar levels of tyrosinase protein [7] and the same has been reported for melanocytes from light Caucasian and black skin [8]. Furthermore, it has been shown that the stimulation of tyrosinase activity and melanin synthesis occurs faster in cytoplasts than in nucleated cells [9]. These results suggest that in some pigment cells tyrosinase is present in a catalytically inactive state and that its activation could be an important control point for melanin synthesis.

Melanin production takes place within specialised intracellular organelles known as melanosomes. Since catalytic domains of tyrosinase are located on the inner side of the melanosomal membrane, its activity could be dependent upon the melanosomal environment. There is evidence that melanosomes are closely related to lysosomes. For instance, both organelles contain the same structural proteins (e.g. lysosome-associated membrane protein, acidic hydrolases, vacuolar type proton pumps) [10,11] and both are affected in genetic disorders such as the Chediak–Higashi and Hermansky–Pudlak syndromes [12,13]. More recently, it has been suggested that the melanosome is accessible to endosomal transport and represents a highly specialised lysosome rather than a completely unique structure [14,15]. This hypothesis is supported by studies which have shown that when human diploid fibroblasts are transfected with tyrosinase and TRP-1, the enzymes co-localise in lysosomes and melanogenesis occurs [16,17].

There are several reports that melanosomes are acidic organelles and when mature can have a pH as low as 4.0 [18,19]. It has been assumed that this low melanosomal pH facilitates melanogenesis [20–22]. However, this is not consistent with the finding that mammalian tyrosinase has optimal enzymatic activity at neutral pH [26–28] and shows little activity at $pH < 5$. On the contrary, there are reports that agents which raise lysosomal pH can increase tyrosinase activity. Thus increases in tyrosinase activity have been reported in pigmented mouse B16 melanoma cells in response to 10 mM NH_4Cl and the proton/metal ion exchanging ionophores, nigericin and monensin [26,27]. This stimulatory activity occurred in intact melanoma cells but not cell lysates, emphasising the importance of intact intracellular membranes. It was proposed that an elevation of melanosomal pH could be responsible for the increase in tyrosinase activity, although other possibilities were not excluded. Ammonium chloride has been shown to inhibit degradation of tyrosinase and increase its half-life by 3-fold [28], and ionophores could act by the facilitating exchange of univalent metal ions.

The purpose of this study was to investigate the effect of increasing melanosomal pH on melanogenesis. It has been shown that the selective vacuolar type H^+-ATPase inhibitors bafilomycin A1 (BafA1) and concanamycin A that target acidic intracellular compartment pH [29,30] are effective in increasing pH in these compartments in melanocytic cells

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Abbreviations: TRP-1 and TRP-2, tyrosinase-related proteins type 1 and 2; Gp100, silver locus 100 kDa protein; MART, melanoma-associated antigen recognised by T cells; BafA1, bafilomycin A1

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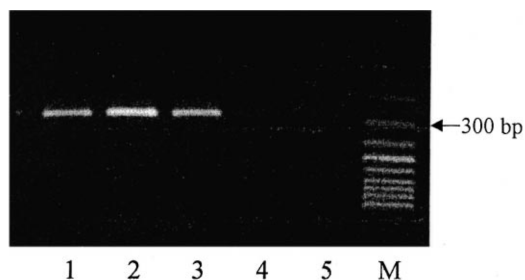

Fig. 1. RT-PCR for human and mouse tyrosinase mRNA. Human melanocytes (positive control), lane 1; B16-G4F, lane 2; FM3, lane 3; FM57, lane 4; FM81, lane 5; 100 bp 'ladder' MW marker, lane M.

(N. Smit, personal communication). In the present study, we examined the effects of BafA1 and concanamycin A in amelanotic but tyrosinase positive melanoma cells.

2. Materials and methods

2.1. Cells and cell cultures

Human melanoma cell lines FM3, FM57 and FM81 established from metastatic melanoma nodules were gifts from Dr A.F. Kirkin. The amelanotic mouse B16-G4F line was a gift from Prof. A. Eberle. Melanoma cells were cultured in RPMI 1640 medium supplemented with foetal bovine serum (10%). BafA1 and cycloheximide were obtained from Sigma, geneticin (G418) from Gibco BRL and concanamycin A from Calbiochem.

2.2. Reverse transcription (RT)-PCR

Total RNA was isolated using TRI Reagent (Sigma). The quality of RNA was tested by the A_{260}/A_{280} ratio and 1.5% agarose gel electrophoresis. cDNA synthesis was performed with MoMLV reverse transcription kit (Fermentas) following the manufacturer's instruction using approximately 1 μg of total RNA and 500 pmol of oligo(dT_{16}) primers in 20 μl of reaction mixture. 1 μl of cDNA was used for PCR amplification in 50 μl of reaction mixture containing: 1×PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 2.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM dNTP (Fermentas) and 1.0 U of recombinant Taq polymerase (Fermentas). The parameters used for the 'touch down' amplification were initially 10 cycles of 94°C for 30 s, 65°C (decreasing 0.5°C per cycle) for 30 s and 72°C for 1 min followed by 25 cycles with the annealing temperature of 60°C. Primers specific for 284 bp fragment of human and mouse tyrosinase were used (forward: TTGGCAGATTGTCTGTAGCC; reverse: AGGCATTGTGCATGCTGCTT). The following primers were used to test human melanoma lines only: 792 bp fragment of TRP-1 (forward: CACAAAGAGCTGCAAACC, reverse: AGGAAGGGAGAAAGAAGG); 873 bp fragment of TRP-2 (forward: CCCTACATCCTACGAAACC, reverse: TTGAGAATCCAGAGTCCC); primers specific for Gp100 and MART were used as described [31].

2.3. Tyrosinase activity

Tyrosinase activity was assayed by measuring the production of radioactive water from tritiated L-tyrosine as described [26]. Briefly, L-[3,5-^{3}H]tyrosine (46 Ci/mmol, from Amersham) was diluted in cell culture medium to produce a final concentration of 5 μCi of L-[3,5-^{3}H]tyrosine per ml. Cells were returned to incubator and 200 μl aliquots of medium were collected after periods of 4, 8 and 24 h. Protein was precipitated by adding 200 μl of 15% (w/v) trichloroacetic acid, and the supernatant treated with 50 mg of Norit A charcoal slurry to remove labeled tyrosine.

2.4. Melanin and protein assays

The amount of melanin was assayed by dissolving a washed cell pellet directly in 1 ml of Soluene 350 (Packard Instruments) and incubating the samples for 2 h at 60°C. Synthetic melanin (Sigma) was used to construct a standard curve for the range of 1–50 μg of melanin per ml of Soluene 350. Melanin absorbance was measured at 475 nm wavelength. Protein concentrations were determined with an assay kit, based on the Lowry assay (Bio-Rad).

3. Results

3.1. Cell characterisation by RT-PCR

Both the B16-G4F and FM3 melanoma cells were found to express tyrosinase mRNA. Although the FM57 and FM81 melanoma cells expressed melanocyte markers Gp100, MART, TRP-1 and TRP-2, they showed no detectable expression of the tyrosinase gene (Fig. 1). The FM3 melanoma cells also expressed Gp100, MART and TRP-2 but no TRP-1.

3.2. Tyrosinase activity and melanin

The levels of tyrosinase activity in FM3 and B16-G4F cells were found to be almost undetectable under control conditions. The FM57 and FM81 melanoma cells that were included as controls contained no tyrosinase activity. No melanin was detected in any of melanoma lines under control conditions.

BafA1 and concanamycin A produced dose-related increases in tyrosinase activity in the tyrosinase positive cell lines FM3 and B16-G4F and as seen in Fig. 2, concanamycin A was the more potent of the two compounds. This order of potency correlates with their respective binding affinities to vacuolar type H^+-ATPase (Calbiochem general catalogue, 2000). Concentrations of both compounds in excess of 20 nM were cytotoxic (data not shown). Submaximal concentrations of 3 nM concanamycin A and 10 nM BafA1 were therefore used in all subsequent experiments and as shown in Fig. 2, these concentrations have similar potencies in stimulating tyrosinase activity. In response to 10 nM BafA1 and 3 nM concanamycin A, cell lines showed a rapid and statistically

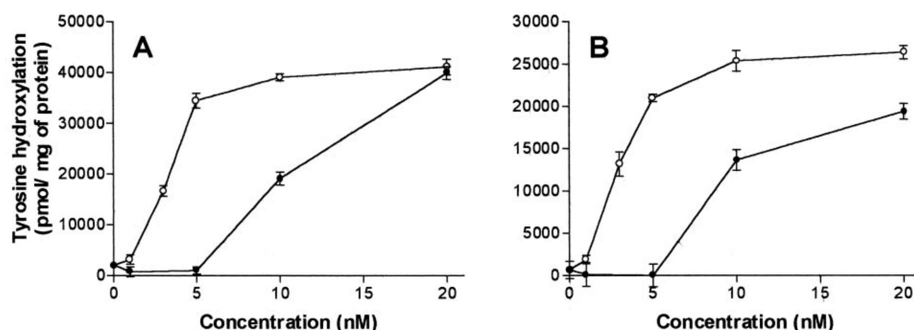

Fig. 2. Effect of vacuolar type H^+-ATPase inhibitors on tyrosinase activity in FM3 (A) and B16-G4F (B) cells. The cells were incubated for 24 h with varying concentrations of concanamycin A (○) and BafA1 (●). Doses higher than 20 nM were toxic for cells.

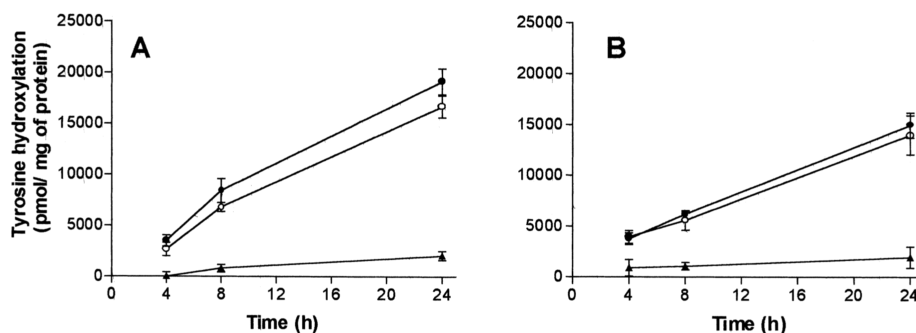

Fig. 3. Time course of tyrosinase activation by vacuolar type H^+-ATPase inhibitors in FM3 (A) and B16-G4F cells (B). The cells were incubated for the indicated periods with: (▲) complete medium (control); (○) 3 nM concanamycin A; (●) 10 nM BafA1.

significant increase in tyrosinase activity which lasted for the 24 h duration of the experiment (Fig. 3). At this time, the cell pellets were visibly darker and contained measurable amounts of melanin (Fig. 4). The protein synthesis inhibitor cycloheximide (2 μg/ml) had no effect on these increases in tyrosinase activity (Fig. 5) and melanin (data not shown). Incubation of the cells with a high dose of cycloheximide (10 μg/ml) alone or the cell cycle arresting agent geneticin (200 μg/ml) failed to induce tyrosinase activity and melanin production (data not shown).

Incubation of the tyrosinase negative FM57 and FM81 melanoma cells with the same concentrations of BafA1 and concanamycin A produced no detectable amounts of [^{3}H]water or melanin.

4. Discussion

A number of studies have suggested that tyrosinase in pigment cells does not necessarily exist in a catalytically optimal state [6,32]. There have also been several reports that activation of this enzyme can occur independently of increases in its synthesis [5,33]. In the present study, the amelanotic, but tyrosinase positive, FM3 and B16-G4F melanoma cells were found to contain almost undetectable levels of tyrosinase activity and intracellular melanin was absent. However, in response to BafA1 and concanamycin A, there was a rapid increase in tyrosinase activity followed by melanin production. The levels of melanin production and tyrosinase activity in the FM3 and B16-G4F cells in response to BafA1 and concanamycin A were comparable to those that we observe in pigmented human melanocytes and B16-F1 mouse melanoma cells, respectively. A high dose of cycloheximide and a cell cycle arresting concentration of geneticin had no effect on melanogenesis, eliminating the possibility that the increases in melanogenesis were associated with non-specific cytostatic effects of BafA1 and concanamycin A. The fact that the melanogenesis response was not abolished by cycloheximide makes it unlikely that tyrosinase abundance was increased and points towards an activation of pre-existing enzyme. A non-genomic mechanism is therefore implicated and this is consistent with the rapid kinetics of tyrosinase activation. Thus our findings support the view that under normal conditions FM3 and B16-G4F melanoma cells contain tyrosinase but because this is inactive, melanin synthesis is suppressed and the cells exhibit an amelanotic phenotype. However, in response to the vacuolar type proton pump inhibitors which induce an increase in the pH of acidic intracellular compartments, tyrosinase is activated and melanin production initiated.

It has previously been reported that tyrosinase activity in vitro is minimal at pH < 5 and that the enzyme is irreversibly inactivated at a pH lower than 4 [23–25]. However, as the pH rises, tyrosinase activity increases, reaching a maximal level at near pH 7.0. This range of proton concentration (pH 5.0–7.0) corresponds well with the variations of pH inside organelles of the endosomal/lysosomal pathway that are created and maintained by proton pumps. As melanosomes are closely related

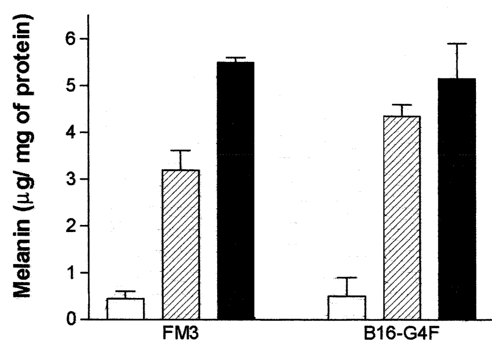

Fig. 4. Melanin production in response to vacuolar type H^+-ATPase inhibitors. Cells were collected after 24 h incubation in complete medium (open bar) containing 3 nM concanamycin A (striped bar) or 10 nM BafA1 (closed bar). The control values are below the level of assay sensitivity (< 0.5 μg/mg of protein).

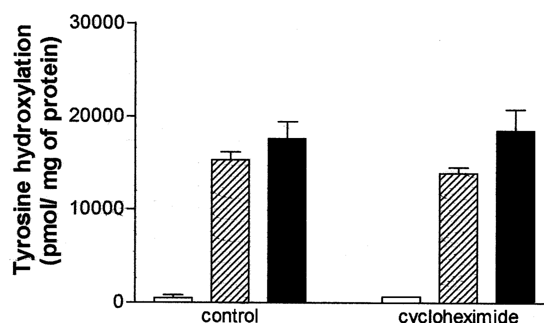

Fig. 5. Effect of cycloheximide on tyrosinase activation by vacuolar type H^+-ATPase inhibitors in FM3 cells. Cells were incubated for 24 h in complete medium (open bar) containing 3 nM concanamycin A (striped bar) or 10 nM BafA1 (closed bar) with or without cycloheximide (2 μg/ml).

to this group of acidic organelles, it is possible that they show similar variations of pH and this could have physiological importance for melanogenesis through a non-genomic control of tyrosinase activity. It is of interest that cyclic AMP, which has been reported to increase lysosomal pH [34], is a well-recognised stimulator of melanogenesis. Melanogenesis also occurs in response to ultraviolet (UV) irradiation and increases have been observed in melanoma cells as early as 1–3 h after UV irradiation [35]. The speed of this response would suggest that non-genomic mechanism operates before de novo synthesis of tyrosinase occurs. Whether this rapid increase in melanin synthesis in response to UV is induced by a change in melanosomal pH remains to be investigated. Nevertheless, our results would suggest that melanosomal pH could function as a 'control switch' providing pigment cells with a fast and dynamic regulatory mechanism for melanogenesis.

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