Glutathione Depletion Increases Tyrosinase Activity in Human Melanoma Cells

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The aim of the present work was to estimate the effect of intracellular glutathione depletion on melanogenesis in human melanoma cells. We determined tyrosine hydroxylation activity, the rate-limiting step of the pathway, and ^{14}C-melanin formation, an assay reflecting the global eumelanogenic pathway. Intracellular glutathione was depleted by treatment with buthionine-S-sulfoximine, a well-known inhibitor of γ-glutamylcysteine synthetase.

The intracellular depletion of glutathione was substantial after 20 h of incubation with 50 μM buthionine-S-sulfoximine, although a significant effect could be observed after 6 h. Tyrosine hydroxylase activity increased in parallel with glutathione depletion, to reach 160% with respect to the control values during 24 h of buthionine-S-sulfoximine treatment. We have found the response to buthionine-S-sulfoximine to be dose dependent and the two different human cell lines HBL and LND1 to have similar, if not identical, responses. ^{14}C-melanin formation assay revealed even greater activation, up to 400% of the control values. This indicates that glutathione depletion may have two distinct effects: first, a direct one on tyrosinase activity and, second, an effect on the promotion of eumelanogenesis. The stimulation of tyrosine hydroxylase can be explained by a possible inactivation of the enzyme by endogenous thiol compounds rather than by a direct effect of buthionine-S-sulfoximine itself on tyrosinase. The data suggest that thiol compounds may play a role for stimulation of melanogenesis by ultraviolet radiation. Key words: glutathione/tyrosinase/melanogenesis.
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The biosynthesis of melanin, the main pigment found in mammalian skin, hair, ears, and eyes, proceeds through a complex series of enzymatic and chemical reactions [1]. The melanins produced are conventionally classified into two major types: eumelanins (brown or black) and sulfur-containing (red or yellow) pheomelanins. Tyrosinase is the key enzyme of the first part of the melanogenesis pathway, catalyzing the conversion of L-tyrosine into L-dopaquinone. This latter o-quinone may subsequently be converted to L-dopachrome, which is a eumelanin intermediate, or alternatively may react non-enzymatically with cysteine or glutathione (GSH) to form the sulfur-containing pheomelanins.

Intramelanocytic GSH is involved in the regulation of melanogenesis in at least two ways: first, by its possible interaction with the tyrosinase-active site [2,3], and second, by the reaction of its thiol group with L-dopaquinone, which may lead to sulphydryl-dopa conjugates and hence the sulfur-containing pheomelanins, instead of eumelanins [2,4,5].

One of the most well-known signals for increased pigment production in human skin is ultraviolet radiation (UVR), although the mechanisms of UVR action remain unknown. Chakraborty *et al* [6] reported that UV light acts indirectly, increasing the number of melanocyte-stimulating hormone (MSH) binding sites on the cell membrane, but Friedmann and Gilchrest [7] showed that UVR can stimulate melanogenesis in the absence of added MSH. Although MSH could be produced endogenously by melanocytes [8], other mediators could also be involved in the cell melanogenic response to UVR. One plausible mediator might be GSH because it is well known that UVR increases extra- and intracellular generation of oxygen radicals, and GSH would react with these radicals and would protect epidermal cells from the cytotoxic effects [9–11] of reactive oxygen.

Buthionine-S-sulfoximine (BSO) inhibits intracellular GSH synthesis [12,13] and has been widely used for depleting intracellular GSH, allowing for the investigation of the role of GSH in more detail. The aim of this study was to examine the role of GSH as a modulator of the melanogenesis pathway in human melanoma cells, particularly its specific effects on tyrosine hydroxylase activity and on melanin formation.

MATERIALS AND METHODS

Reagents Radioactive L-[3,5-^{3}H]-tyrosine (specific activity 47 Ci/mmol) and L-[^{14}C]-tyrosine (specific activity, 479 mCi/mmol) were obtained from Amersham (U.K.). BSO, GSH, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Janssen Chimica (Belgium). Nicotinamide adenine dinucleotide (NADPH) tetrasodium salt and yeast glutathione reductase (120 U/ml) were purchased from Boehringer Mannheim (Germany). L-tyrosine, trichloroacetic acid, HCl, Norit A, Celit 535, and ethylenediamine tetraacetic acid (EDTA) were obtained from Merck (Germany) and 3,4 dihydroxyphenylalanine (L-dopa) and Nonidet P40 were from Sigma Chemical Co (St. Louis, MO). Culture media and supplements were purchased from Gibco (U.K.).

Cells HBL and LND1 are human cell lines established in our laboratory and cultured in Ham's F10 medium containing 10% fetal calf serum, 1% of a penicillin-streptomycin solution containing 10,000 U/ml–10,000 μg/ml, 1% of kanamycin (1,000 μg/ml), and 1% glutamine (200 mM) [14].

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Abbreviations: BSO, buthionine-(S,R)-sulfoximine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

Cells were seeded at 6×10^6 cells per 175-cm^2 flask. After 24 h culture, 1–50 μM BSO was added to the medium for various times and cells were harvested by trypsin-EDTA treatment. In all treatments, cell viability was higher than 90%. A triplicate set of flasks was used for every experiment. Tyrosinase was extracted from 8.5×10^6 cells in 1 ml Hanks' balanced salt solution after homogenization and sonication. Crude extracts were divided into two lots: one left as such for protein determination (Lowry *et al* [15]) and eumelanin formation as described below and the other treated with Nonidet P40 at 1% final concentration and centrifuged at $10{,}000 \times g$ for 10 min. Tyrosine hydroxylase activity and GSH concentration were measured in the supernatants as described below.

In some experiments, HBL cells were solubilized in Hank's balanced salt solution containing 1% Nonidet P40 and suspended at 4×10^6 cells/ml. The preparation was ultracentrifuged at $105{,}000 \times g$ for 30 min in a TL-100 Beckman ultracentrifuge to remove non-solubilized material. The supernatant was divided into 2-ml aliquots and incubated with 10, 2, or 0 mM freshly prepared GSH in 10 mM phosphate buffer, pH 6.8, containing 0.1% Nonidet P40 for 15 min. Each aliquot was dialyzed twice at 4°C during 3 h against 500 ml of the same buffer. After 3 h the dialysis buffer was changed to remove GSH excess. Tyrosine hydroxylase activity was measured immediately after dialysis. The experiment was repeated twice.

Tyrosine Hydroxylase Assay and Melanin Formation Assay Incubations were performed as described by Hearing and Ekel [16], as modified by Jara *et al* [17].

Briefly, tyrosine hydroxylase was conducted as follows: 30 μl cell extract were added to 10 μl of 0.25 mM L-tyrosine (final concentration) containing 0.0204 mM tritiated L-tyrosine (specific activity 49 Ci/mmol) and 10 μl of a solution containing chloramphenicol (1 mg/ml), penicillin G (1000 units/ml), bovine serum albumin (0.1 mg/ml), and 0.05 mM L-dopa (the cofactor), in a final volume of 50 μl. Antibiotics were included to inhibit any protein synthesis and/or incorporation of radioactive tyrosine into macromolecules other than melanin. All solutions were prepared in 10 mM phosphate buffer, pH 6.8. The assays were run in triplicates in Eppendorf tubes at 37°C for 1 h. After incubation, 50 mg Norit A, 50 mg Celit 535 and 450 μl of 1% trichloroacetic acid were added. Tubes were shaken for 30 min and centrifuged at $13000 \times g$. Supernatant (100 μl) was removed and counted for radioactivity in a β counter. One unit of tyrosine hydroxylase was defined as the amount of enzyme that catalyzes the hydroxylation of 1 μmol L-tyrosine/min.

Thirty microliters of crude extract were used to measure melanin formation. Incubations were performed at 37°C in microtiter plates for 1 h as for tyrosine hydroxylase (same substrate and same cofactor final concentrations), but ^{14}C-tyrosine was used as the radioactive substrate at the concentration of 0.106 mM (specific activity 479 mCi/mmol). The reaction was stopped by the addition of 150 μl of a solution containing 6% trichloroacetic acid and 10 mM unlabeled tyrosine. The samples were passed through fiberglass filters and the filters were washed three times with 150 μl 0.1 mM HCl, dried, and counted. Blanks were subtracted from experimental samples to obtain net cpm. One unit of tyrosinase was considered as the amount of enzyme able to catalyze the incorporation of 1 μmol L-tyrosine into eumelanin (acid insoluble)/min, as described by Hearing and Ekel [16].

GSH Determination Total intracellular GSH was measured in cell extract containing 1% Nonidet P40 following the method of Griffith [18]. This assay is based on the reduction of the aromatic DTNB to chromogenic thio-nitrobenzoic acid (TNB) by GSH, which is oxidized to the disulfide form (GSSG). Total GSSG was then reduced back to GSH by glutathione reductase and NADPH. Routinely, 50 μl of cell extracts were diluted to 200 μl with phosphate buffer (10 mM, pH 7.5; 6.3 mM EDTA), and mixed with 100 μl of a 6 mM DTNB solution and 700 μl of NADPH (0.3 mM in phosphate buffer, pH 7.5; 10 mM, 6.3 mM EDTA) at 25°C. After the sample reached a stable absorbance value, the reaction was started with 10 μl of a 20-U/ml glutathione reductase solution and the increase in absorbance was monitored at 412 nm. Two nmol of GSH per ml measured by this method corresponds approximately to 1 mM intracellular GSH concentration.

RESULTS

Fourteen experiments showed that treatment of HBL human melanoma cells with 50 μM BSO for 24 h increased tyrosine hydroxylase activity. Statistical analysis of these experiments revealed an increase of approximately 158% in tyrosine hydroxylase activity (control, 0.863 ± 0.315; BSO treated, 1.36 ± 0.50 mU/mg, $0.001 < p < 0.01$), and a significantly higher increase in ^{14}C-melanin formation of about 400% (control, 30.9 ± 20.8 μU/mg; BSO treated, 122.4 ± 81.5 μU/mg, $p < 0.001$). The BSO-induced increase of tyrosine hydroxylase activity was concentration dependent and very similar in two different human melanoma cell lines, HBL and LND1 (Fig 1). Therefore, all subsequent following experiments were performed using HBL cells.

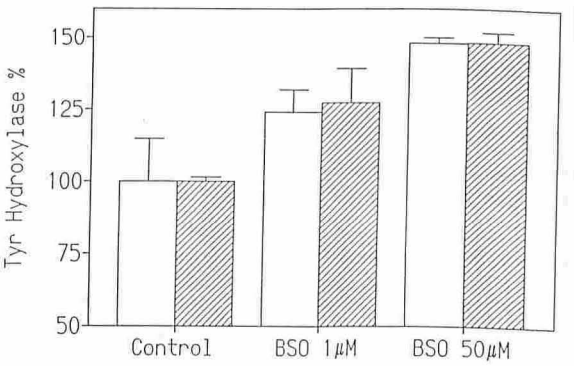

Figure 1. Effect of BSO on tyrosine hydroxylase. HBL *(empty bars)* and LND1 *(dashed bars)* human melanoma cells were exposed to 50 μM BSO for 24 h. Enzyme activity was measured according to Jara *et al* [17]. Control values were calculated as mean ± SD (n = 3): 0.675 ± 0.109 mU/mg for HBL and 0.864 ± 0.013 mU/mg for LND1. This assay has been repeated twice on each cell line with similar results.

Figure 2 illustrates the time-course change in GSH intracellular concentration, tyrosine hydroxylase activity (Fig 2*a*), and ^{14}C-melanin formation (Fig 2*b*) after cell exposure to 50 μM BSO. GSH concentration fell 3 h after the beginning of the incubation and reached the lowest values at 20 h after treatment. Tyrosine hydroxylase activity increased rapidly between 6 and 12 h (Fig 2*a*). ^{14}C-melanin formation increased with time for at least 48 h (Fig 2*b*).

When cell extracts were incubated for 15 min with this thiol compound and then extensively dialyzed to remove excess free GSH, a concentration-dependent decrease in enzyme activity of 27% or 88% for 2 or 10 mM GSH, respectively, was observed (Fig 3). The results support the hypothesis that GSH inactivates tyrosinase.

To test the possibility that the effect of BSO on tyrosinase activity is mediated by GSH depletion, HBL cells were exposed to 10 mM exogenous GSH with or without 50 μM BSO (Fig 4*a*). Both tyrosine hydroxylase activity and ^{14}C-melanin formation were decreased after incubation with GSH alone ($0.001 < p < 0.01$ and $p < 0.001$, respectively). However, the combined GSH plus BSO treatment also provoked a decrease of tyrosine hydroxylase very similar to when GSH alone was applied (about 40% with respect to untreated cells, $0.001 < p < 0.01$). In contrast, the combined effect of BSO plus GSH enhanced ^{14}C-melanin formation twofold, compared with untreated cells. Furthermore, intracellular GSH increased after incubation with exogenous GSH alone, but it was depleted by BSO irrespective of the presence or absence of exogenous GSH (Fig 4*b*).

DISCUSSION

In our experiments, as previously reported [19,20], BSO treatment induced GSH depletion in human melanoma cells. When 50 μM BSO was added to the culture media, intracellular GSH content reached a minimum level after 20 h of exposure in HBL cells (Fig 2*a*), a period similar to that reported by Karg *et al* [19] on IGR1 human melanoma cells. This suggests a greater resistance to GSH depletion in human melanoma cells compared to mouse melanoma cells, in which 25 μM BSO and only 4 h incubation were enough to substantially deplete intracellular GSH [21].

It is well documented that melanogenesis is affected by thiol compounds [2–4,22–26]. This effect is caused by at least two different mechanisms. First, low-molecular-weight thiol compounds can inhibit melanogenesis by direct interaction of thiol groups with the tyrosinase active site [2,3], thus inhibiting tyrosine hydroxyla-

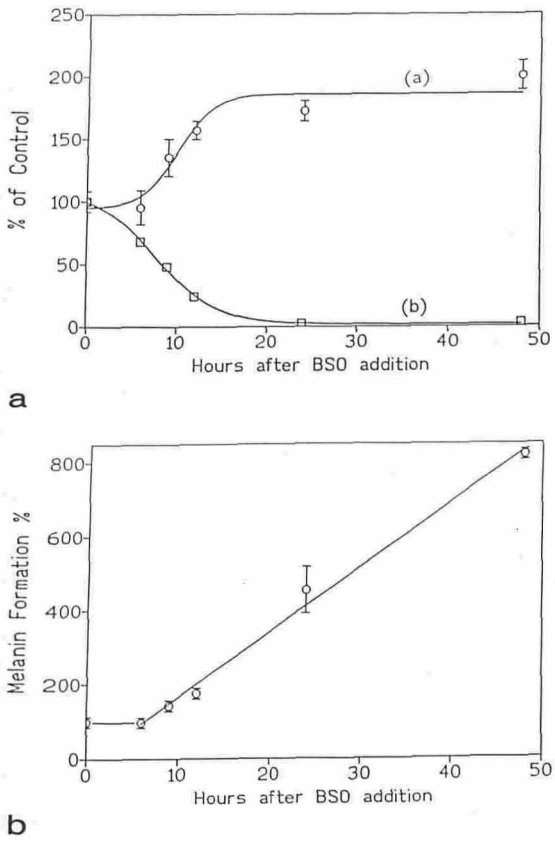

Figure 2. *(a)* Time course of GSH content and tyrosine hydroxylase activity (percent of control) in HBL human melanoma cells treated with 50 μM BSO. GSH (□) was measured according to Griffith [18], and tyrosine hydroxylase (○) was measured according to Jara *et al* [17]. The results are expressed in percent of control. Control values: GSH, mean, 168 nmol/mg (range: 170–166 nmol/mg) (n = 2); tyrosine hydroxylase, 1.21 ± 0.02 mU/mg (mean ± SD; n = 3). The experiment was performed twice with similar results. *(b)* Time course of ^{14}C-melanin formation activity. The activity was measured according to Jara *et al* [17]. Other conditions are presented in *(a)*. Control, 41.4 ± 3.2 μU/mg (mean ± SD; n = 3). This experiment was performed twice with similar results.

tion. Second, thiol groups are able to react with L-dopaquinone to form dopa-thiol conjugates [1], which are pheomelanogenic precursors. The slower rate of the chemical reactions involved in the polymerization of these intermediates and their higher solubility [27] in comparison to that of indols in the eumelanin pathway leads to an apparent inhibition of melanin synthesis, as measured by the ^{14}C-melanin formation assay [2].

Our studies show that BSO treatment promotes the activation of tyrosinase activity in human melanoma cells. This increase might be explained either by a direct activation of the enzyme or by the removal of some endogenous inhibitor. Direct activation of the enzyme is unlikely, as we have observed that BSO had no effect on purified tyrosine hydroxylase activity *in vitro* (data not shown). Accordingly, the effect of BSO on tyrosine hydroxylase activity must be related to the concomitant GSH depletion (Fig 2*a*).

Recently, Jergil *et al* [3] indicated that thiol compounds inactivated tyrosinase. In agreement with their findings, we found that the decrease in enzyme activity induced by incubation with GSH was not recovered by extensive dialysis (Fig 3). The concentration-dependent inactivation of tyrosinase by GSH may be caused by interaction with the copper ion in such a way that GSH could not easily be displaced from the active site of the enzyme.

Considering these results and the normal GSH concentrations in human melanoma cells (1–10 mM), we conclude that the tyrosinase pool may be partially inactivated by endogenous GSH. Thus, a significant part of the increased BSO induced in enzymatic activity

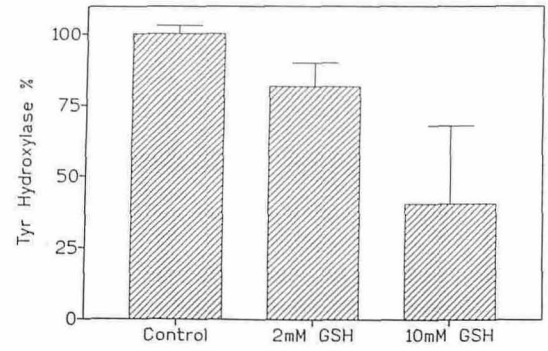

Figure 3. Recovery of tyrosine hydroxylase activity (percent of control) after incubation with GSH and dialysis. HBL cell extracts were preincubated for 15 min with GSH (2 and 10 mM) and then dialyzed as described in *Materials and Methods*. Control value, 0.687 ± 0.048 mU/mg. The experiment was repeated twice with different HBL cell extracts, and the presented results are the mean ± SD from both experiments (n = 4).

would come from *de novo* synthesis of tyrosinase molecules. The low GSH levels measured in BSO-treated cells may prevent the inactivation of the newly synthesized tyrosinase in those treated cells. The reported half-life of tyrosinase in mammalian melanocytes, around 8–10 h [28,29], agrees with the time needed by BSO treatment to increase the tyrosine hydroxylase activity.

As discussed above, the higher rate in ^{14}C-melanin formation compared to the increase in tyrosine hydroxylase results from the effect of GSH depletion not only on tyrosinase, but on the whole pathway. Under physiologic conditions, intracellular GSH levels as well as other thiol compounds should be high enough to favor pheomelanogenesis so that cysteinyl-dopa (the major pheomelanin precursor) is directly [4] or indirectly formed from glutathionyl-dopa hydrolysis [26]. Following intracellular GSH depletion, tyrosine hydroxylation was enhanced, suggesting that melanogenesis flow increases along with L-dopaquinone formation. On the other hand, the ability of L-dopaquinone to react with thiol and yield thiol-dopa conjugates is slower under conditions of GSH depletion. The final result should be that L-dopachrome and hence eumelanogenesis are increased. *In vivo* this mechanism may be autocatalytic, because dopa is generated in the eumelanin pathway by spontaneous reaction between dopaquinone and cyclodopa [1,17]. This dopa could act as cofactor for tyrosinase and thereby activate tyrosine hydroxylation.

Intracellular BSO-induced GSH depletion was expected to be reversed by adding exogenous GSH to the cell culture medium. When GSH was added alone, intracellular GSH increased, but more interestingly, when BSO and GSH were added together, intracellular GSH remained very low and very similar to the GSH content found in cells treated with BSO alone (Fig 4*b*). These data are in agreement with the proposal by Meister *et al* [11,12], who postulated that exogenous GSH cannot be taken up by mammalian cells, but must be spliced by the membrane-bound transpeptidase. Therefore, in the presence of BSO, γ-glutamyl-cysteine synthetase is inhibited and subsequently prevents GSH synthesis. However, the cytosolic concentrations of GSH precursors, such as cysteine, would be greater than in untreated cells [4]. This same cysteine may be responsible for preventing an increase in tyrosine hydroxylase when both GSH and BSO are present, as it is a tyrosinase-inactivating agent and has more affinity for the enzyme catalytic site than does GSH [3].

Although tyrosine hydroxylase activity was actually decreased, treatment BSO plus GSH increased ^{14}C-melanin formation. However, the increase was lower than the one provoked by BSO alone (Fig 4*a*). This apparent discrepancy between tyrosine hydroxylase and ^{14}C-melanin formation data can be partially explained by the action of thiol compounds on the pheo/eumelanogenesis switch discussed above. In addition, ^{14}C-melanin assay reflects the global eumelanogenesis pathway, in which other melanogenic enzymes

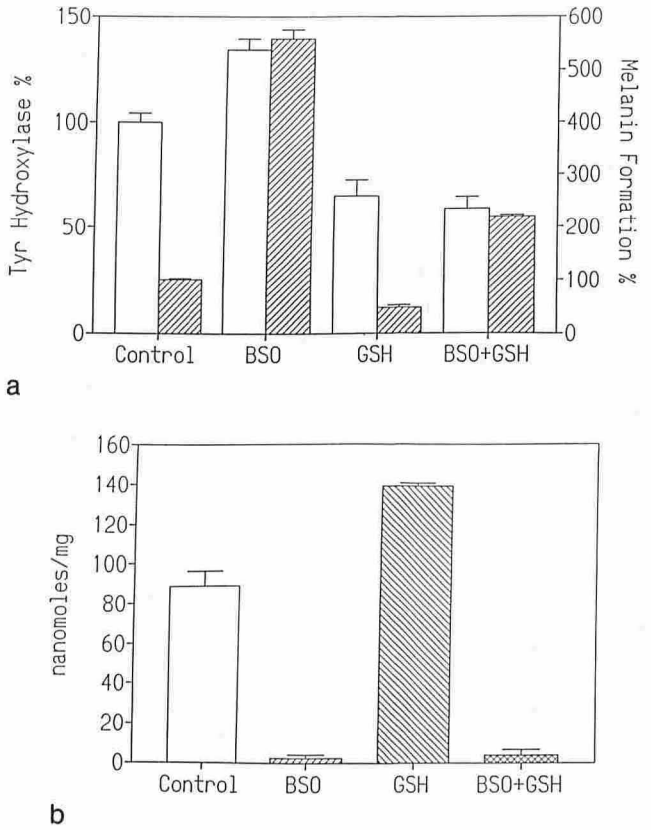

Figure 4. *(a)* Tyrosine hydroxylase activity *(empty bars)* and ^{14}C-melanin formation *(dashed bars)* in HBL cells treated with GSH (10 mM) and BSO (50 μM) for 24 h. Control values (mean ± SD; n = 3): 1.07 ± 0.04 mU/mg (tyrosine hydroxylase) and 47.0 ± 1.9 μU/mg (^{14}C-melanin assay). *(b)* Intracellular GSH content of HBL cells. Cultures were treated as described in *(a)*. Control mean: 210 nmol/mg (range: 208–212, n = 2). This experiment was performed twice with similar results.

different from tyrosinase [29], such as TRP-1 and TRP-2, could also play important roles in the modulation of this assay [16]. In agreement with this possibility, we recently reported that BSO increases TRP-2 activity in B16 mouse melanoma cells [21], and the same enzyme has been shown to accelerate ^{14}C-melanin formation [30]. Taking all these considerations together, we conclude that ^{14}C-melanin formation may be enhanced even when tyrosine hydroxylase is inhibited.

Although it is well known that UVR induces melanogenesis, it is not clear whether melanocytes are stimulated either directly by UVR or indirectly by other factors produced in the skin that mediate the UVR action. The role of α-MSH and cyclic adenosine monophophate (cAMP) in the process is under discussion [6,7], but other possible intracellular mediators whose concentrations can be affected by UVR could be involved. GSH plays a major role in protecting human skin from the free-radical damage induced by UVR [10], and the drop in the intramelanocytic GSH induced by UVR might be an inducer of the cellular pigment response. Our data suggest that GSH depletion increases tyrosinase activities and favors eumelanogenesis (versus pheomelanogenesis). Further studies are needed to elucidate the influence of UVR on the concentration of GSH in melanocytes.

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