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ORIGINAL ARTICLE

Glycine hydroxamate inhibits tyrosinase activity and melanin contents through downregulating cAMP/PKA signaling pathways

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Abstract Among the eight amino acid hydroxamates tested, Glycine hydroxamate (GH) was the best inhibitor of mushroom tyrosinase (TYR). With L-tyrosine as substrate, the GH inhibition of the monophenolase activity of the mushroom TYR was noncompetitive. GH decreased not only TYR protein expression, but also melanin content, tyrosinase-related protein (TRP)-1, TRP-2, and microphthalmia-associated transcription factor (MITF) expression in B16F10 melanoma cells while in the presence of α-melanocyte-stimulating hormone (α-MSH). GH also significantly decreased the isobutylmethylxanthine (IBMX)-induced increase in melanin content, which was not prevented by the ERK inhibitor PD98059. These results suggest GH has the potential for use in cosmetic hypopigmentation.

Keywords Glycine hydroxamate · Hypopigmentation · Isobutylmethylxanthine · Melanin, microphthalmia-associated transcription factor · Protein kinase A · Tyrosinase

Abbreviations

AAHs	Amino acid hydroxamates
TYR	Tyrosinase
MITF	Microphthalmia-associated transcription factor
α-MSH	α-Melanocyte-stimulating hormone
IBMX	Isobutylmethylxanthine
CREB	Cyclic AMP response element binding protein
PKA	Protein kinase A
TRP-1	Tyrosinase-related protein-1
TRP-2	Tyrosinase-related protein-2
ERK	Extracellular signal-regulated kinases

Introduction

The use of mushroom tyrosinase (TYR; EC 1.14.18.10) in the study of melanin formation and its inhibition and activation by natural or synthetic compounds was recently reviewed by Seo et al. (2003). TYR is a Cu-containing metalloenzyme, which in melanocytes catalyzes the rate-limiting reactions of both monophenol hydroxylation to *o*-diphenol [i.e., tyrosine is catalyzed to produce dihydroxyphenylalanine (DOPA)] and *o*-diphenol oxidation into the corresponding *o*-quinone (i.e., DOPA is further oxidized to dopaquinone). The dopaquinone is further processed by tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2) into eumelanin (which has a brown or black color) or non-enzymatically polymerized into pheomelanin (which has a yellow to reddish-brown color) (Hearing and Ekel 1976; Hearing and Tsukamoto 1991). Abundant

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production and excessive activation of tyrosinase can cause various dermatological disorders such as melasma, freckles, age spots, and senile lentigines (Gilchrest et al. 1996). TYR inhibitory activity was reported for arbutin, aloesin, hydroquinone, kaempferol, kojic acid, and resveratrol (Briganti et al. 2003), and for some peptides (Schurink et al. 2007; Abu Ubeid et al. 2009).

In mammalian melanocyte cells, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) cascade is one of the most important pathways in the regulation of melanogenesis (Busca and Ballotti 2000). The α-melanocyte-stimulating hormone (α-MSH) and other cAMP-elevating agents such as isobutylmethylxanthine (IBMX) and forskolin activate adenylate cyclase, resulting in an increase in intracellular cAMP concentration and the activation of the PKA cascade (Halaban et al. 1983). PKA phosphorylates the cAMP-responsive binding protein (CREB) at Ser-133, which in turn activates the promoter of microphthalmia-associated transcription factor (MITF) (Ao et al. 1998). In particular, the melanocyte-specific transcriptional factor MITF-M supports the expression of melanogenesis-related genes, TYR and TYR-related proteins 1 and 2 (TRP-1 and TRP-2) (Yasumoto et al. 1997). In addition, cAMP-elevating agents may inhibit the extracellular signal-regulated kinases (ERK) to reduce MITF degradation and stimulate melanogenesis-related gene expression (Englaro et al. 1998).

The hydroxamic acid (R-CONHOH moiety) derivatives were reported to have pharmacological and biological side effects that resulted in cancer, cardiovascular disease, Alzheimer's disease, and tuberculosis (Muri et al. 2002). L-Aspartic acid β-hydroxamate and L-glutamic acid γ-hydroxamate were reported to exhibit antioxidant and angiotensin-converting enzyme inhibitory activities (Liu et al. 2004), while hydroxyurea exhibited antioxidant and amine oxidase inhibitory activities (Liu et al. 2010). The nicotinic acid hydroxamate showed higher anti-monophenolase and anti-diphenolase activities of mushroom TYR than kojic acid, and also showed anti-pigmentation activity in murine melanoma B16F10 cells (Lin et al. 2012a, b).

In this study, eight commercially available amino acid hydroxamates (AAHs), namely: DL-alanine hydroxamate (AH), L-arginine hydroxamate (RH), L-aspartic acid β-hydroxamate (DH), L-glutamic acid γ-hydroxamate (EH), glycine hydroxamate (GH), L-lysine hydroxamate (KH), DL-serine hydroxamate (SH), and DL-threonine hydroxamate (TH) were used to compare the anti-monophenolase and anti-diphenolase activities of mushroom TYR to that of arbutin. The anti-melanogenic effects of the AAHs were then investigated, as well as possible signaling pathways in murine melanoma B16F10 cells.

Materials and methods

Materials

AAHs (AH, DH, EH, GH, KH, RH, SH, and TH), arbutin, L-DOPA, glycine (Gly), L-glutamine (Gln), α-MSH, IBMX, mushroom TYR, radio-immunoprecipitation assay (RIPA) buffer, and L-tyrosine (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-β-actin antibody, horseradish peroxidase-conjugated goat anti-mouse, and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against TYR, TRP-1, and TRP-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against MITF, CREB, and p-CREB were from Abcam Inc. (Cambridge, MA, USA). Murine melanoma B16F10 cells (BCRC 60031) were purchased from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan).

TYR inhibitory activity by spectrophotometric assays

The TYR inhibition assay was performed by spectrophotometric method, essentially as described by Jiménez et al. (2001) but with some modifications. Each 200-μL reaction mixture contained: 12.5 mM of each AAH or different concentrations of GH and Gly (1.25–12.5 mM); 5.0 mM L-tyrosine (for monophenolase activity assay) or 5 mM L-DOPA (for diphenolase activity assay); and mushroom TYR (1,000 μ/mL) in 250 mM of phosphate buffer (pH 6.8). Each reagent was sequentially added to a 96-well microtiter plate. Arbutin (1.25–12.5 mM) was used as a positive control, and distilled water was added in lieu of a test sample as a blank. The absorbance was measured at 490 nm (for monophenolase activity) or 475 nm (for diphenolase activity) using an ELISA reader (TECAN Sunrise microplate reader; Männedorf, Switzerland) for 60 min and expressed as absorbance changes at either 490 nm (ΔA_{490}) or 475 nm (ΔA_{475}). The TYR inhibitory activity was calculated using the following equation: $(\Delta A_{blank} - \Delta A_{sample}) \div \Delta A_{blank} \times 100$ %. IC_{50} indicates the concentration for half-inhibition against TYR.

TYR inhibitory activity by activity stains

The TYR inhibitory assay was also performed by TYR activity stains in 10 % native PAGE gels, as described by McMahon et al. (2007) with some modifications. Each assay contained TYR (10 units) in 250 mM phosphate buffer (pH 6.8) and was either pretreated with one of the AAHs (30 mM), left untreated, or pre-mixed with a concentration of GH and Gly (5–40 mM). Incubation was

overnight at 4 °C. After electrophoresis, the gel was divided into half and pre-equilibrated with 100 mM phosphate buffer (pH 6.8) for 30 min. Each portion of gel was then placed in a solution of L-tyrosine or L-DOPA (10 mM) in a 37 °C water bath for staining. The dark brown band against the clear background showed the position of monophenolase or diphenolase activity, respectively. Untreated TYR was used as a control. The TYR inhibitory activity of each AAH was quantified and expressed as a relative density (% area) using the Syngene G: bBOX imaging system (Syngene, UK) equipped with the GeneSnap software (Syngene, UK); the relative density of untreated TYR was set at 100 %.

Kinetic analysis of TYR inhibition

The kinetic parameters of mushroom TYR (100 units) in the presence of arbutin or GH (0.5–2 mM) were calculated from Lineweaver–Burk plots using different concentrations of L-tyrosine (0.125–1.25 mM) as substrates. The velocity was expressed as absorbance changes at 490 nm (ΔA_{490}) per minute.

Cell culture and cell viability assay

The B16F10 melanoma cells were cultured following the methods of Lin et al. (2012b). Different concentrations of GH, Gly and arbutin (0.5–3.5 mM) were added to a seeded 24-well microtiter plate and incubated in a humidified incubator at 5 % CO_2 and 37 °C for 24 h. Five microliter of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL) was added under light protection and after 4 h, 100 μL of DMSO was added to dissolve the formed formazans. The absorbance at 600 nm was measured by an ELISA reader (Sunrise, TECAN, Männedorf, Switzerland) for cell viability assays (Wang et al. 2007).

Determination of cellular TYR activity and melanin content

The B16F10 cells were treated with different concentrations of GH, Gly and arbutin (1 to 3.5 mM) and the addition of α-MSH (50 nM) in a humidified incubator at 5 % CO_2 and 37 °C for 24 h. To determine TYR activity, the cells were harvested and lysed by a cold RIPA buffer containing a protease inhibitor cocktail (P-8340, Sigma Chemical Co.). The protein content of the lysates was quantified using the bicinchoninic acid protein assay kit (Pierce Biotechnology, IL, USA). The TYR inhibitory assay was performed using 50 μL 10 mM L-DOPA and 50 μL of cell lysates, with equal amounts of proteins in a 250 mM phosphate buffer (pH 6.8). Each reagent was sequentially added to a 96-well microtiter plate. The absorbance values at 475 nm were compared against a standard curve of mushroom TYR. To determine melanin content in B16F10 cells, the extraction and analysis were performed as per the previous report (Lin et al. 2012b).

Western blot analysis

B16F10 melanoma cells were treated for 10 or 24 h with GH (1.5–3 mM) in a humidified incubator at 5 % CO_2 and 37 °C, with the addition of either α-MSH (50 nM) or IBMX (100 μM). After electrophoresis, the Western blot analysis was performed as previously reported (Lin et al. 2012b) using the primary antibodies against β-actin, MITF, CREB, and p-CREB (1000-fold dilution by 0.25 % gelatin in NaCl/EDTA/Tris (NET) solution) and TYR, TRP-1, TRP-2 (500-fold dilution by 0.25 % gelatin in NET solution). The inhibitory effects of GH treatment on protein expression were quantified and expressed as a relative density (% area) using the Syngene G:bBOX imaging system (Syngene, UK) equipped with the Gene Snap software (Syngene, UK). The relative density without a tested compound treatment (the control) was set at 100 %.

Determination of cAMP

B16F10 cells were treated with different concentrations of GH (2.5, 3, or 3.5 mM) in a humidified incubator at 5 % CO_2 and 37 °C for 10 h with the addition of IBMX (100 μM). Cells were lysed and the AMP levels in the supernatant were determined using a cAMP Enzyme Immunoassay kit (Cayman Chemical, Switzerland).

Statistical analyses

Values were presented as mean ± SD of three independent experiments and analyzed using one-way ANOVA, followed by the post hoc Tukey's test for multiple mean comparisons. Values not sharing the same alphabetic letter at the same substrate used were significantly different ($P < 0.05$). Student's t test was used for comparison between two treatments. A difference between the control (no tested sample addition) and each treatment was considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

Results

Effects of AAHs on mushroom TYR followed by spectrophotometry and activity stains

AAHs at the same concentrations of 12.5 and 30 mM, respectively, were used in spectrophotometric (Fig. 1a)

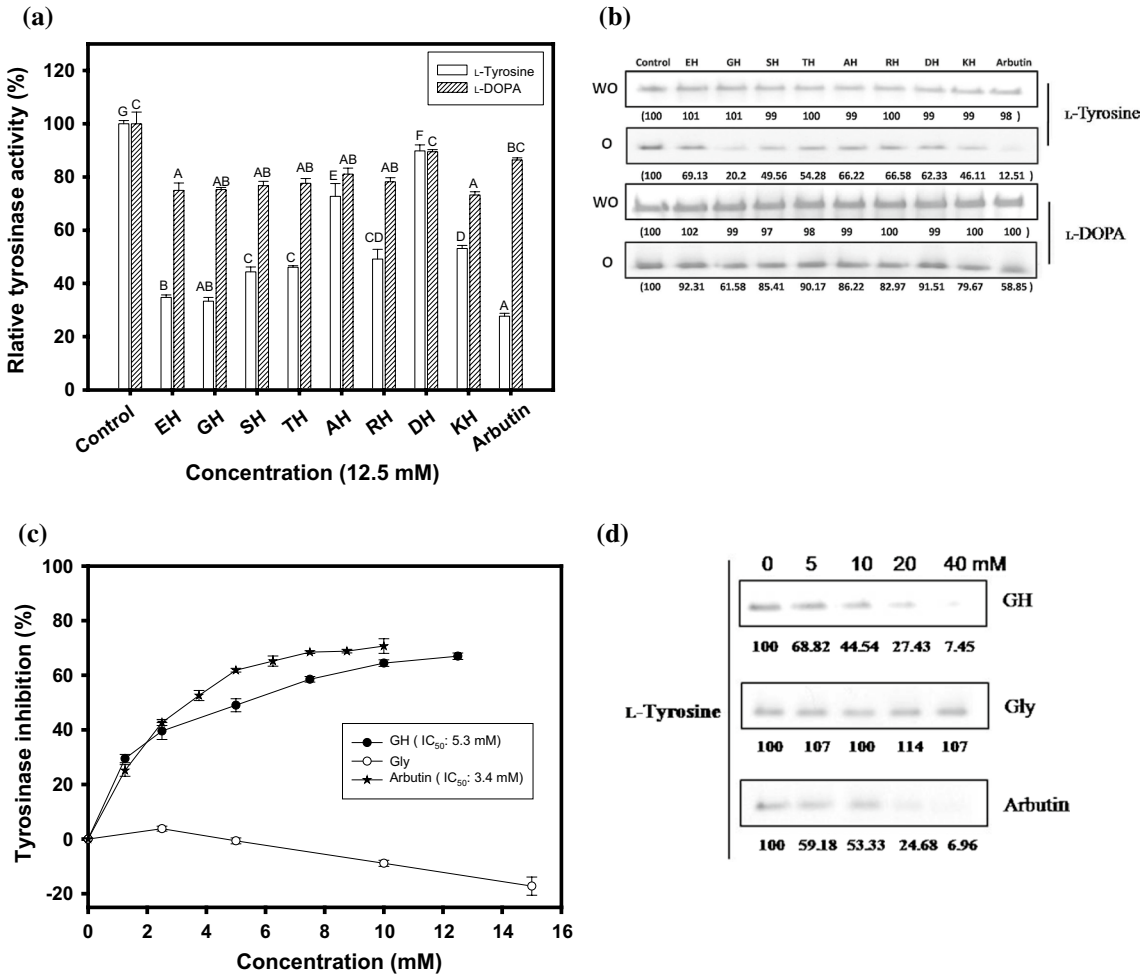

Fig. 1 Effects of amino acid hydroxamates on monophenolase activity (L-tyrosine as substrates) or diphenolase activity (L-DOPA as substrates) of mushroom tyrosinase by **a** spectrophotometric methods (tested compounds at 12.5 mM) and expressed as relative tyrosinase activity (%); and by **b** activity stains (tested compounds at 30 mM) 10 % native PAGE gels without overnight (WO) or overnight (O) pretreatment and expressed as relative tyrosinase activity (%). The dose effects of glycine hydroxamate (GH), glycine, and arbutin on mushroom tyrosinase activity by **c** spectrophotometric methods and **d** activity stains. Values not sharing the same alphabetic letter at the same substrate used were significantly different ($P < 0.05$)

and activity stains (Fig. 1b) for anti-TYR inhibitory assays. In anti-monophenolase activity assays using L-tyrosine as a substrate (Fig. 1a), the tested AAHs showed significant differences compared to the control ($P < 0.05$). The tested AAHs can be divided into three groups by residual TYR activities. The first group (arbutin, GH and EH) was 27.69, 33.38 and 34.75 %; the second group (SH, TH, RH and KH) was 44.38, 46.11, 49.14 and 53.16 %, and the last group (AH and DH) was 72.76 % and 89.80 % of the respective control. Using L-DOPA for anti-diphenolase assays (Fig. 1a), the inhibitory activities of the AAHs were less than those shown for anti-monophenolase. With the exception of DH and arbutin, the tested AAHs showed significant differences compared to the control ($P < 0.05$). Without overnight pretreatment (WO), the AAHs showed no anti-TYR activity staining (Fig. 1b) using either L-tyrosine or L-DOPA as a substrate. With overnight pretreatment (O), the anti-monophenolase activity of the tested AAHs can be divided into three groups by the residual TYR activities. The first group (arbutin and GH) was, respectively, 12.51 and 20.2 %; the second group (KH, SH, and TH) was 46.11, 49.56, and 54.28 %; and the last group (DH, AH, RH and EH) was 62.33, 66.22, 66.58, and 69.13 % of the control. Using L-DOPA for anti-diphenolase activity stains (Fig. 1b), the inhibitory effects of the AAHs were less than that for anti-monophenolase, and only GH (61.58 % of the control) achieved results close to arbutin (58.85 %).

Based on the above findings, the anti-monophenolase activity of GH was further studied using the spectrophotometric assay (Fig. 1c) and activity stains (Fig. 1d). It was found that GH showed dose-dependent anti-monophenolase activity at a concentration for half-inhibition (IC_{50})

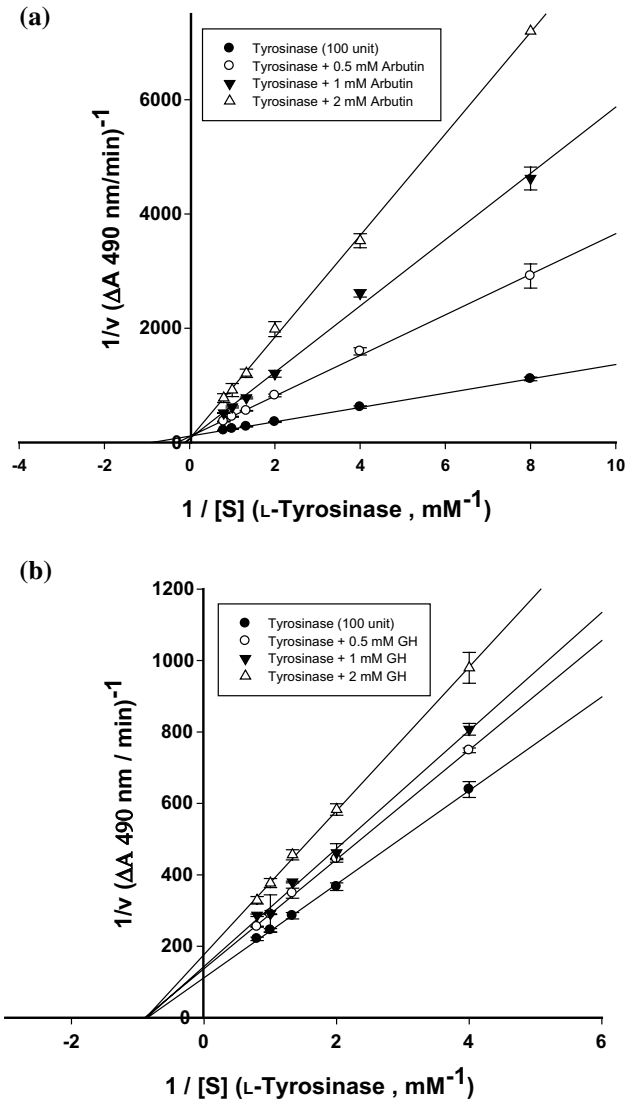

Fig. 2 The kinetic parameters of monophenolase activity of mushroom tyrosinase in the presence of 0.5, 1, and 2 mM **a** arbutin or **b** glycine hydroxamate (GH)

of only 5.3 mM (0.48 mg/mL) compared to the 3.4 mM (0.93 mg/mL) required for arbutin. Conversely, glycine showed an activation of monophenolase activity. GH and arbutin, but not Gly, also showed dose-dependent inhibition of monophenolase by activity stains (Fig. 1d). The calculated IC_{50} of GH and arbutin was 0.8 mg/mL (8.88 mM) in the spectrophotometric assay and 3.04 mg/mL (11.16 mM) in the stains assay (Fig. 1d).

Kinetic analysis of TYR inhibition

The K_m and V_{max} for TYR were determined as 0.072 mM and 0.0084 (ΔA490/min) of L-tyrosine, respectively, using the Lineweaver–Burk plot (Fig. 2). In the presence of 0.5, 1, and 2 m Marbutin, the K'_m, respectively, increased to 0.89, 2.33 and 11.93 mM, while the V_{max} and V'_{max} were the same, showing that arbutin acted as a competitive inhibitor. In the presence of 0.5, 1, and 2 mM GH (Fig. 2b), the V'_{max}, respectively, decreased to 0.0077, 0.0068 and 0.0062 (ΔA490/min), while K_m and K'_m remained the same, showing that GH acted as a noncompetitive inhibitor.

GH-induced hypopigmentation in B16F10 cells

GH, Gly and arbutin (0.5–3.5 mM) showed no apparent cytotoxicity toward B16F10 cells in the presence of α-MSH (Fig. 3a), however, GH and arbutin also showed dose-dependent reductions and significant differences in the melanin content (Fig. 3b) and TYR activities (Fig. 3c) compared to the control. The reduced melanin levels were positively correlated with the lowered TYR activity in GH-treated or arbutin-treated cells. These results showed that GH exhibited anti-hyperpigmentation effects comparable to arbutin in B16F10 cells. Using the immune stains, the melanogenic enzyme expressions—including those of TYR, TRP-1, TRP-2 and MITF—were quantified and normalized with β-actin (Fig. 3d). The results demonstrated that GH exhibited dose-dependent inhibition of TYR, TRP-1, TRP-2, and MITF protein expression in B16F10 cells.

Effect of GH on cAMP levels and protein expressions in cAMP/PKA signaling pathways of B16F10 cells

To elucidate the possible signaling pathway of GH-induced hypopigmentation, IBMX was used to induce melanogenesis with or without GH co-treatments (Fig. 4). With the addition of IBMX (Fig. 4a), TYR protein expression increased up to 2.6-fold (from 100 to 258.7 %). GH co-treatment reduced TYR expression to about 45 % (258.7 to 115.4 %), which was almost the same as the GH treatment without any IBMX additions (100 to 44.2 %). GH showed clear dose-dependent reduction of melanin levels in the presence of IBMX (Fig. 4b); with the addition of the specific MEK inhibitor PD98059, the melanin content was almost the same as in IBMX-treated B16F10 cells. Thus, PD98059 clearly did not reverse the GH-induced hypopigmentation in the presence of IBMX. On the other hand, cAMP levels in B16F10 cells (Fig. 4c) were significantly increased after IBMX treatments ($^{\#}P < 0.05$). Co-treatment with GH showed significant dose-dependent differences and decreased cAMP content compared to samples treated with IBMX only. These results suggested that while the MEK/ERK signaling pathway was not mainly involved, the cAMP/PKA pathway might be involved in GH-induced hypopigmentation. Therefore, the key proteins of cAMP/PKA signaling pathways, including CREB, phospho-CREB, TYR, and MITF were investigated using

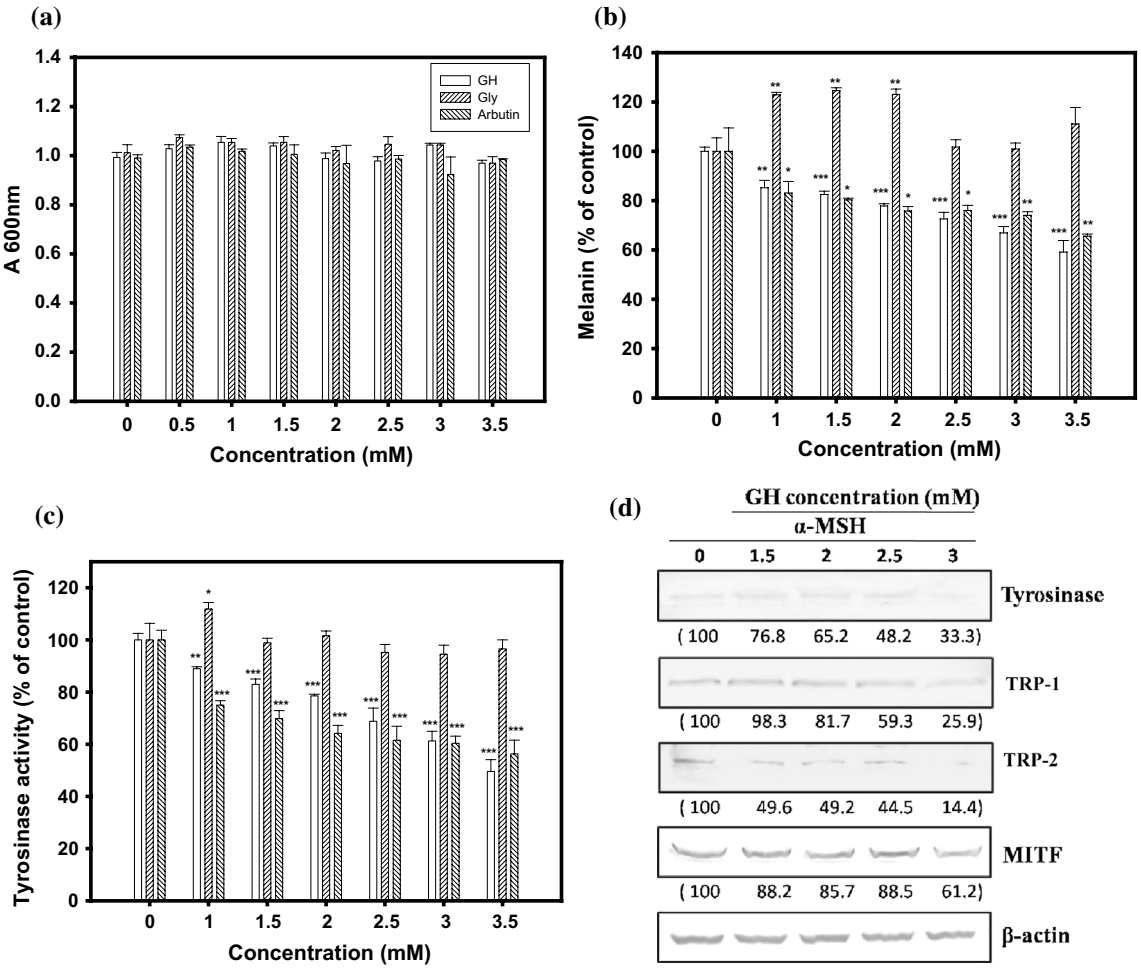

Fig. 3 Dose effects (0.5–3.5 mM) of glycine hydroxamate (GH), glycine (Gly), and arbutin on melanogenesis of B16F10 cells for **a** cell viability, **b** melanin contents, **c** tyrosinase activity, and **d** protein expressions of melanogenic enzyme (tyrosinase, TRP-1, TRP-2) and MITF in the presence of α-MSH (50 nM). Student's *t* test was used for comparison between the untreated control (the left column) and treatment. A difference was considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$

Western blot analysis (Fig. 4d). GH treatments showed dose-dependent inhibition against CREB, p-CREB, TYR, and MITF protein expression in the presence of IBMX. These results suggested that GH might reduce hyperpigmentation by lowering cAMP levels enough to affect the cAMP/PKA signaling pathway, ultimately downregulating TYR and MITF protein expression via the CREB protein expression and phosphorylation.

Discussion

This is the first report of GH exhibiting not only anti-TYR activity and anti-melanogenic enzyme expression, but also melanin reduction results comparable to arbutin. This may have applicable use in cosmetics. Hirsch and Kaplan (1961) described the conversion of hydroxamic acids to the corresponding amides by mouse liver homogenates. Several naturally occurring hydroxamic acid derivatives from fungi, actinomycetes, yeast, bacteria and plants are related structurally to amino acids (Neilands 1967). The results reveal that these AAHs show greater inhibition of monophenolase activity of mushroom TYR than diphenolase (Fig. 1). TYR was proposed to have different catalytic sites for L-tyrosine and L-DOPA (Hearing and Tsukamoto 1991) and our data support this hypothesis. GH showed both the highest TYR inhibition and also dose-dependent inhibition among the tested AAHs. The IC_{50} of GH reached mM levels; however, the weight percent concentration for half-inhibition of GH was calculated to be less than that of arbutin (Fig. 1c, d).

Both EH and GH showed anti-monophenolase activity, however, only GH showed TYR inhibitory activity by overnight (O) pretreatments on the native PAGE gels. The hydroxamic acid moiety of amino acid derivatives alters the intricate acid–base properties that might influence the TYR inhibitory activities. The aminohydroxamic acids are

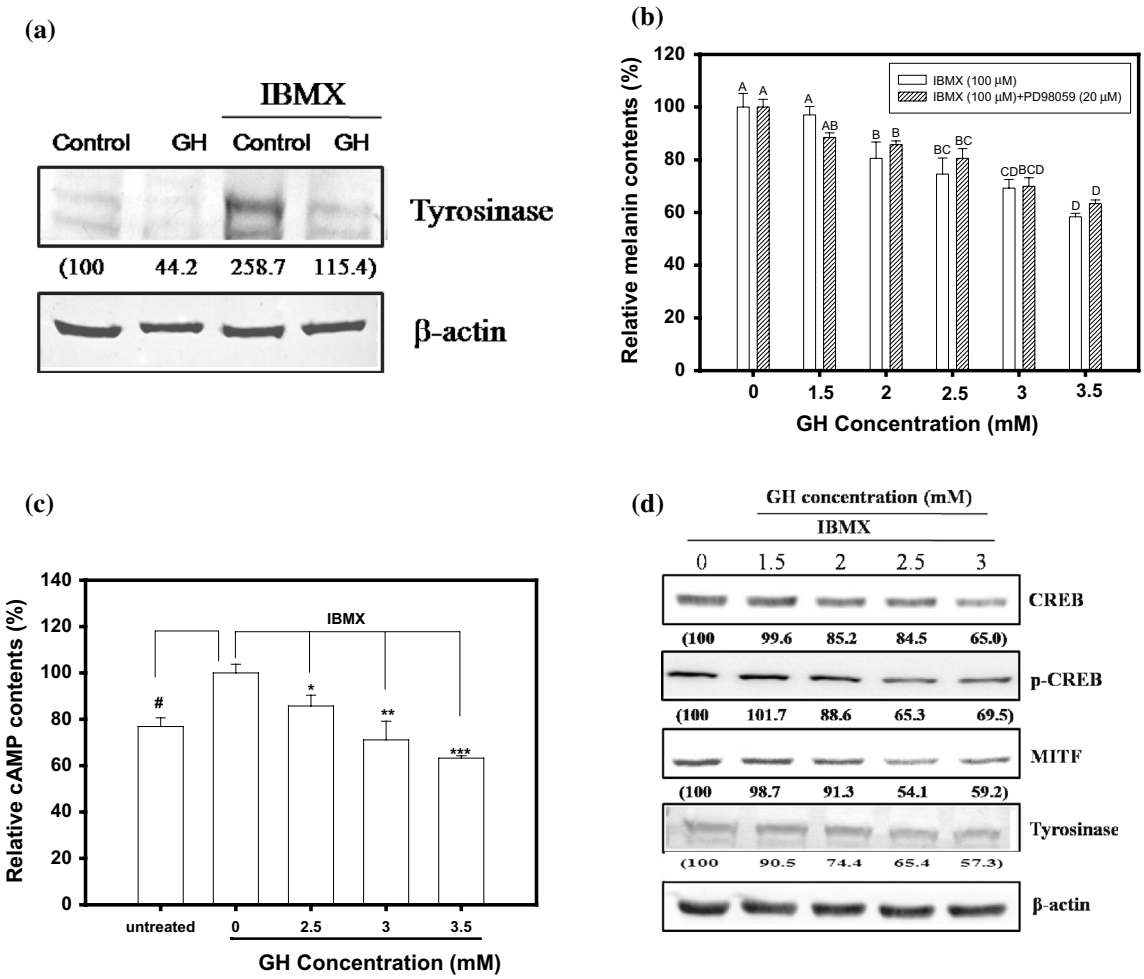

Fig. 4 With or without 100 μM IBMX additions, **a** 3.5 mM glycine hydroxamate (GH) on tyrosinase protein expressions by western blotting; and **b** dose effects (1.5–3.5 mM) of GH on melanin contents without or with PD98059 (20 μM) co-treatment for 24 h. **c** Dose effects (2, 3, and 3.5 mM) of GH on cAMP productions. **d** Dose effects (1.5, 2, 2.5, and 3 mM) of GH on downregulation of the CREB, phosphorylated CREB, MITF, and tyrosinase protein expressions. Values not sharing the same alphabetic letter at the same treatment were significantly different ($P < 0.05$). Student's t test was used for comparison, and a difference between the control and blank was considered statistically significant when $^{\#}P < 0.05$; a different between the control and each treatment was considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$

characterized by the presence of an amino group ($–NH_2$) and a hydroxamic acid group (–CO–NH–OH) separated by a spacer and, therefore, have at least two proton dissociation constants (pKa). For GH, the pKa was reported to be 7.42 (NH group of hydroxamate) and 9.18 (α-amino group), and for EH (the glutamic acid γ-hydroxamate) the constants are 2.14 (α-carboxyl group), 8.59 (NH group of hydroxamate), and 9.63 (α-amino group) (Tegoni and Remelli 2012). At a low pH and in the absence of metal ions, GH exists in the protonated form ($–NH_3^+$, –CO–NH–OH). As pH increases, GH is deprotonated to form $–NH_3^+$, –CO–N^-–OH and at a high pH, it exists in the deprotonated form ($–NH_2$, –CO–N^-–OH). At a low pH and in the absence of metal ions, EH exists in the protonated form (–COOH, $–NH_3^+$, –CO–NH–OH). As pH increases, EH is deprotonated to form $–COO^-$, $–NH_3^+$, –CO–NH–OH, eventually forming $–COO^-$, $–NH_3^+$, –CO–N^-–OH and ultimately, when at a high pH, its deprotonated form $–COO^-$, $–NH_2$, –CO–N^-–OH. Schurink et al. (2007) proposed that peptides containing negatively charged residues of aspartic acid or glutamic acid were usually unfavorable for TYR interactions, and the residual TYR activities depended on tested peptide electrostatic binding and the catalytic site's inhibitory effects on TYR. We propose that GH and EH influence tyrosine binding to TYR and exhibit inhibitory activities at short assay time when using the spectrophotometric method. However, prolonged incubation of EH with TYR results in the loss of its anti-TYR potential, possibly due to the functional group deprotonation, as with the inhibition of Ni-containing urease (Kobashi et al. 1975).

Arbutin showed competitive inhibition against monophenolase activity in the current study as well as in a previous report (Tomita et al. 1990). However, GH showed noncompetitive inhibition of monophenolase activity, meaning that it could bind to TYR far from the catalytic sites and alter conformations to reduce TYR activities with or without L-tyrosine. On the other hand, GH has been reported to form complexes or metallacrowns with Cu (II) ions and to act as a powerful chelator for many metal ions (Fernandes et al. 1997; Tegoni and Remelli 2012), which might contribute in part to its inhibitory activities against the Cu-containing TYR.

It was reported that the free amino acids L-Ala, L-Leu, L-Ile and Gly—but not D-Ala, D-Leu, or D-Ile—showed a concentration-independent decrease of melanin production in B16F0 melanoma cells. However, none of these amino acids had significant inhibitory activity against mushroom TYR (Ishikawa et al. 2007). Our results showed that under non-cytotoxic concentrations, treatment by GH could dose dependently inhibit TYR activity and reduce the melanin content to be comparable to that of arbutin in B16F10 melanoma cells; with the addition of α-MSH, GH-treated B16F10 cells could reduce the protein expression of melanogenic enzymes (TYR, TRP-1 and TRP-2) and MITF. We propose that GH might down-regulate MITF protein expression, which in turn inhibits the expression of TYR, TRP-1 and TRP-2, ultimately resulting in the retardation of melanogenesis. We also found that GH induces a dose-dependent decrease in melanin content and reduces TYR protein expression in B16F10 cells while in the presence of IBMX. The cAMP/protein kinase A signaling pathway is one of the most important in the induction of MITF expression, which up-regulates melanogenic enzymes at both mRNA and protein levels (Busca and Ballotti 2000). The α-MSH could increase the intercellular cAMP concentration by binding to the melanocortin 1 receptor (MC1R), a G protein-coupled receptor, while IBMX increases the intracellular cAMP levels by inhibiting cAMP phosphodiesterase (PDE) (Ao et al. 1998; Conti and Beavo 2007).

In melanocytes and melanoma cells, elevation of the intracellular cAMP content resulted in the activation of the Ras/ERK cascade, leading to an inhibition of tyrosinase expression and melanogenesis; the specific MEK inhibitor PD98059 increased tyrosinase expression and activity (Englaro et al. 1998). The current results showed that the ERK inhibitor PD98059 did not reverse the GH-induced hypopigmentation in the presence of IBMX, and the MEK/ERK cascade might not participate in GH-induced anti-melanogenesis. However, cAMP levels in GH-treated B16F10 cells showed a dose-dependent decrease with IBMX additions. Furthermore, levels of CREB, phospho-CREB, TYR, and MITF protein expression were reduced by GH treatments in the presence of IBMX. Therefore, it was suggested that the anti-pigmentation effects of GH might down-regulate cAMP production and consequently decrease levels of CREB and phosphorylated CREB, resulting in a decrease in MITF transcription and protein expression. In addition, the elevation of the intracellular cAMP content also resulted in the activation of the MEK/ERK cascade.

In conclusion, our results show that GH might contribute to the hypopigmentation effects. Hydroquinone, kojic acid, and arbutin are popular anti-pigmentation agents, widely used in the cosmetic industry, but some studies have raised concerns about the adverse effects and carcinogenic potential of some of the common whitening agents (Lau et al. 2001; Blaut et al. 2006). GH could achieve hypopigmentation effects similar to those of arbutin but at a lower concentration, suggesting its potential use in cosmetic whitening and necessitating further investigation.

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Conflict of interest The authors declare that they have no conflict of interest.

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