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Approaches to Identify Inhibitors of Melanin Biosynthesis via the Quality Control of Tyrosinase

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Tyrosinase, a copper-containing glycoprotein, is the rate-limiting enzyme critical for melanin biosynthesis in specialized organelles termed melanosomes that are produced only by melanocytic cells. Inhibitors of tyrosinase activity have long been sought as therapeutic means to treat cutaneous hyperpigmentary disorders. Multiple potential approaches exist that could control pigmentation via the regulation of tyrosinase activity, for example: the transcription of its messenger RNA, its maturation via glycosylation, its trafficking to melanosomes, as well as modulation of its catalytic activity and/or stability. However, relatively little attention has been paid to regulating pigmentation via the stability of tyrosinase, which depends on its processing and maturation in the endoplasmic reticulum and Golgi, its delivery to melanosomes and its degradation via the ubiquitin-proteasome pathway and/or the endosomal/lysosomal system. Recently, it has been shown that carbohydrate modification, molecular chaperone engagement, and ubiquitylation all play pivotal roles in regulating the degradation/stability of tyrosinase. While such processes affect virtually all proteins, such effects on tyrosinase have immediate and dramatic consequences on pigmentation. In this review, we classify melanogenic inhibitory factors in terms of their modulation of tyrosinase function and we summarize current understanding of how the quality control of tyrosinase processing impacts its stability and melanogenic activity.

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INTRODUCTION

Melanin synthesis in melanocytic cells is ultimately regulated by tyrosinase (EC 1.14.18.1), a membrane-bound copper-containing glycoprotein, which is the critical rate-limiting enzyme. Tyrosinase is produced only by melanocytic cells and following its synthesis and subsequent processing in the ER and Golgi, it is trafficked to specialized organelles, termed melanosomes, wherein the pigment is synthesized and deposited. In the skin and hair, the melanosomes are transferred from melanocytes to neighboring keratinocytes and are distributed in those tissues to produce visible color. Excess melanin production or abnormal distribution can cause irregular hyperpigmentation of the skin. In order to develop therapies or prophylactics that improve or prevent hyperpigmentary disorders, such as melasma, and age spots, disruption of tyrosinase activity has usually been targeted. Many approaches to achieving such decreases exist, for example, by competitive or non-competitive inhibition of tyrosinase catalytic activity, by disruption of tyrosinase maturation or by decreasing its stability, any of which would reduce melanin synthesis and deposition. Potential avenues to manipulate tyrosinase levels include the modulation of tyrosinase messenger RNA (mRNA) transcription and its post-transcriptional stability.

Levels of intracellular proteins are regulated by a balance between their synthesis and degradation, which is also true for tyrosinase. In contrast to effects on most proteins, reduced stability and function of tyrosinase has dramatic results on ensuing pigmentation. Tyrosinase is degraded endogenously, at least in part, by proteasomes (Halaban *et al.*, 1997), multicatalytic proteinase complexes that selectively degrade intracellular ubiquitylated proteins, such as abnormal proteins misfolded in the endoplasmic reticulum (ER) and short-lived proteins (Hiller *et al.*, 1996; Hochstrasser, 1996; Hershko and Ciechanover, 1998). Recent studies have revealed that oligosaccharide trimming of asparagine (*N*)-linked glycans, molecular chaperone-induced folding, and ubiquitylation as a sorting determinant are closely connected to the proteolysis machinery of tyrosinase, however, little attention has been paid to regulating tyrosinase degradation as an approach to control melanogenesis. This review examines recent studies correlating the quality control of tyrosinase and its stability, and their implications in controlling melanin biosynthesis.

MULTIPLE APPROACHES TO INHIBIT MELANIN SYNTHESIS

Inhibitors of tyrosinase activity have been reviewed previously (Mishima *et al.*, 1988; Briganti *et al.*, 2003; Slominski *et al.*, 2004; Kim and Uyama, 2005; Wang and Hebert, 2006; Solano *et al.*, 2006). Many targets exist for

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Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; mRNA, messenger RNA; OCA, oculocutaneous albinism; PTU, phenylthiourea; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; TYRP1, tyrosinase-related protein-1

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Table 1. Mechanistic classification of the melanogenesis inhibitory factors via tyrosinase modulation

Mechanism	Substance	Specificity[1]	Reference[2]
Inhibition of tyrosinase mRNA transcription			
	5-Bromodeoxyuridine	General	Kidson and DeHaan, 1990
	TPA[3]/insulin	General	Fuller *et al*., 1990
	TGF-β1[4]	General	Martínez-Esparza *et al*., 1997
	TNF-α[5]	General	Martínez-Esparza *et al*., 1998
	Agouti signal protein	Specific	Aberdam *et al*., 1998
	Hydrogen peroxide	General	Jiménez-Cervantes *et al*., 2001
	Ceramide	General	Kim *et al*., 2002
	Dihydrolipoic acid/lipoic acid	General	Lin *et al*., 2002
	Sphingosine-1-phosphate	General	Kim *et al*., 2003
	Lysophosphatidic acid	General	Kim *et al*., 2004b
	(−)-Epigallocatechin-3-gallate/hinokitiol	General	Kim *et al*., 2004a
	Terrein	General	Park *et al*., 2004b
	Piperlonguminine	General	Kim *et al*., 2006b
	Sphingosylphosphorylcholine	General	Kim *et al*., 2006a
Aberration of tyrosinase glycosylation			
	Glucosamine/tunicamysin	General	Imokawa and Mishima, 1982
	Glutathione	General	Imokawa, 1989
	Feldamycin[6]	General	Terao *et al*., 1992
	N-Butyldeoxynojirimycin	General	Petrescu *et al*., 1997
	Calcium D-pantetheine-*S*-sulfonate	General	Franchi *et al*., 2000
	Ferritin	General	Maresca *et al*., 2006
Inhibition of tyrosinase catalytic activity			
	Phenylthiourea	Specific	Dubois and Erway, 1946
	Hydroquinone	Specific	Jimbow *et al*., 1974
	Azelaic acid	Specific	Nazzaro-Porro *et al*., 1979
	Kojic acid[7]	Specific	Mishima *et al*., 1988
	Dithiothreitol	General	Naish-Byfield *et al*., 1994
	Arbutin[8]	Specific	Maeda and Fukuda, 1996
	Magnesium L-ascorbyl-2-phosphate	General	Kameyama *et al*., 1996
	2-*O*-α-D-Glucopyranosyl-L-ascorbic acid	General	Kumano *et al*., 1998
	α-Tocopheryl ferulate	General	Funasaka *et al*., 1999
	4-Tertiary butylphenol	Specific	Yang and Boissy, 1999
	Bathocuproein disulphonate	Specific	Petris *et al*., 2000
	Ellagic acid	Specific	Shimogaki *et al*., 2000
	Aloesin[9]	Specific	Jones *et al*., 2002
	Bisindolylmaleimide	General	Park *et al*., 2004a
	4,4′-Dihydroxybiphenyl	General	Kim *et al*., 2005c
	4-*n*-Butylresorcinol	Specific	Kim *et al*., 2005a
Acceleration of tyrosinase degradation			
	TGF-β1[4]	General	Martínez-Esparza *et al*., 1997
	TNF-α[5]	General	Martínez-Esparza *et al*., 1998
	Linoleic acid	General	Ando *et al*., 1999
	2,2′-Dihydroxy-5,5′-dipropyl-biphenyl	General	Nakamura *et al*., 2003

Table 1 continued on following page

Table 1. continued

Mechanism	Substance	Specificity[1]	Reference[2]
	TPA[3]/phospholipase D2	General	Kageyama *et al.*, 2004
	25-Hydroxycholesterol	General	Hall *et al.*, 2004
	Phenylthiourea	Specific	Hall and Orlow, 2005

TGF, transforming growth factor; TPA, 12-*O*-Tetradecanoylphorbol-13-acetate; TNF, tumor necrosis factor.
[1]Factors that regulate many physiological proteins in addition to tyrosinase are listed as 'general', whereas factors that specifically affect tyrosinase or melanocyte regional proteins are listed as 'specific'.
[2]Representative reports are cited.
[3]12-*O*-Tetradecanoylphorbol-13-acetate.
[4]Transforming growth factor-β1.
[5]Tumor necrosis factor-α.
[6]BMY-28565.
[7]5-Hydroxy-2-(hydroxymethyl)-4-pyrone.
[8]β-D-Glucopyranoside of hydroquinone.
[9]2-Acetonyl-8-β-D-glucopyranosyl-7-hydroxy-5-methyl chromone.

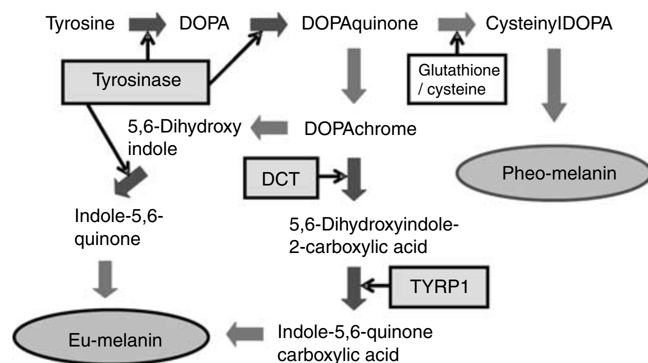

Figure 1. Melanin synthetic pathway and the involvement of melanogenic enzymes. Initial melanin synthesis is catalyzed by tyrosinase and is then divided into eumelanogenesis or pheomelanogenesis. The other melanogenic enzymes, that is, L-3,4-dihydroxyphenylalanine (DOPA) chrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1), are involved in eumelanogenesis, and no specific enzymes have been found that are involved in pheomelanogenesis so far.

controlling melanin synthesis via the regulation of tyrosinase, for example, the transcription of its mRNA, its maturation via asparagine-linked oligosaccharide processing, the modulation of its catalytic activity, and/or its degradation. Suppression of melanin production by melanocytes would be an effective approach to treat a variety of hyperpigmentary disorders. To date, many agents that can inhibit tyrosinase function or melanin synthesis and which lead to decreased melanin production have been reported. Among those, representative substances that have been characterized for their inhibition of tyrosinase function are listed in Table 1. Although three enzymes are known to be involved in melanin biosynthesis in mammals, that is, tyrosinase, tyrosinase-related protein-1 (TYRP1) and dopachrome tautomerase (also known as TYRP2) (Figure 1) (Kobayashi *et al.*, 1994), tyrosinase is the focus of this review as it directly regulates the amount of melanin produced, whereas the other enzymes simply modify the type of melanin synthesized.

Inhibition of tyrosinase mRNA transcription

Melanin synthesis is directly regulated by the enzymatic function of tyrosinase and thus by transcription of its encoding gene. Decreases of tyrosinase mRNA levels in cultured melanoma cells can be elicited by incubation with the thymidine analog 5-bromodeoxyuridine (Kidson and DeHaan, 1990), by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (Fuller *et al.*, 1990; Kuzumaki *et al.*, 1993; Ando *et al.*, 1995), by transforming growth factor-β1 (TGF-β1) (Martinez-Esparza *et al.*, 1997) and by tumor necrosis factor-α (TNF-α) (Martinez-Esparza *et al.*, 1998). Microphthalmia-associated transcription factor, a transcription factor with a basic-helix-loop-helix-zipper motif, is the master regulator of melanogenesis-related gene expression (Tachibana *et al.*, 1996). A number of factors that decrease levels of mRNAs encoding tyrosinase and/or microphthalmia-associated transcription factor in cultured melanoma cells, melanocytes or melanoblasts, including agouti signal protein (Aberdam *et al.*, 1998), hydrogen peroxide (Jimenez-Cervantes *et al.*, 2001), ceramide (Kim *et al.*, 2002), dihydrolipoic acid (Lin *et al.*, 2002), sphingosine-1-phosphate (Kim *et al.*, 2003), lysophosphatidic acid (Kim *et al.*, 2004b), (−)-epigallocatechin-3-gallate and hinokitiol (Kim *et al.*, 2004a), terrain (Park *et al.*, 2004b), piperlonguminine (Kim *et al.*, 2006b), and sphingosylphosphorylcholine (Kim *et al.*, 2006a). Such factors reduce levels of tyrosinase function and melanin synthesis via microphthalmia-associated transcription factor-dependent mechanisms.

Aberrant tyrosinase maturation

Tyrosinase is a glycoprotein with six N-linked glycosylation sites that are conserved in human and mouse tyrosinases (Kwon *et al.*, 1987; Müller *et al.*, 1988; Ujvari *et al.*, 2001). Mutations in the gene encoding tyrosinase are responsible for type 1 oculocutaneous albinism (OCA1) and mutations of critical N-linked glycosylation sites reduce its catalytic function (Branza-Nichita *et al.*, 2000). Aberration of tyrosinase glycosylation in the ER or Golgi inhibits its folding and

maturation and results in hypopigmentation. An early study revealed that glycosylation inhibitors, such as glucosamine and tunicamycin, inhibit melanin synthesis in cultured melanoma cells with no apparent decrease in tyrosinase protein (Imokawa and Mishima, 1982). Other factors have since been shown to inhibit melanogenesis in cultured melanoma cells or in melanocytes by modulating the glycosylation of tyrosinase, for example, glutathione (Imokawa, 1989), ferritin (Maresca *et al.*, 2006), feldamycin (Terao *et al.*, 1992), and calcium D-pantetheine-*S*-sulfonate (Franchi *et al.*, 2000). Studies on N-linked oligosaccharide processing of tyrosinase have demonstrated that *N*-butyl-deoxynojirimycin, an inhibitor of the ER-processing enzymes α-glucosidases I and II, completely blocks the catalytic activity of tyrosinase in B16 melanoma cells with little appreciable change in tyrosinase protein (Petrescu *et al.*, 1997, 2000; Branza-Nichita *et al.*, 1999).

Inhibition of tyrosinase catalytic activity

Numerous reports have described the inhibition of tyrosinase activity, many of them using mushroom tyrosinase as a model. However, studies using mushroom tyrosinase have been omitted from this review owing to limited space and because the regulation of mushroom tyrosinase differs significantly in several respects from mammalian tyrosinase (Pomerantz, 1963; Hearing *et al.*, 1980). Some compounds that are active against mushroom tyrosinase do not show comparable results with mammalian tyrosinase (Galindo *et al.*, 1987; Jacobsohn and Jacobsohn, 1992; Funayama *et al.*, 1995). Inhibitors of tyrosinase function can be divided into two groups, competitive inhibitors and non-competitive inhibitors. Early studies revealed that hydroquinone and azelaic acid, both being potent therapies for hyperpigmentary disorders (Arndt and Fitzpatrick, 1965; Grimes, 1995), are competitive inhibitors of tyrosinase activity as well having cytotoxic effects on melanocytes (Jimbow *et al.*, 1974; Nazzaro-Porro and Passi, 1978; Nazzaro-Porro *et al.*, 1979; Palumbo *et al.*, 1991). Arbutin, a β-D-glucopyranoside derivative of hydroquinone, also inhibits tyrosinase activity competitively but at non-cytotoxic concentrations in cultured melanocytes (Maeda and Fukuda, 1996). Competitive inhibition can also result from treatment of cultured melanocytes, melanoma cells or purified tyrosine with 4-tertiary butylphenol (Yang and Boissy, 1999), aloesin (Jones *et al.*, 2002), 4,4′-dihydroxybiphenyl (Kim *et al.*, 2005d), and 4-*n*-butylresorcinol (Kim *et al.*, 2005a).

Tyrosinase activity depends on the binding and function of two copper atoms at the active site (Furumura *et al.*, 1998; Branza-Nichita *et al.*, 1999) which is facilitated by the Menkes disease-related protein, a copper transporter (Petris *et al.*, 2000). Therefore, chelating copper inhibits the activity of purified or recombinant tyrosinase, for example, by phenylthiourea (PTU) (Dubois and Erway, 1946), by 5-hydroxy-2-(hydroxymethyl)-4-pyrone, also termed kojic acid (Mishima *et al.*, 1988), by dithiothreitol (Naish-Byfield *et al.*, 1994), by bathocuproein disulphonate (Petris *et al.*, 2000), or by ellagic acid (Shimogaki *et al.*, 2000). Another non-competitive method of inhibiting tyrosinase activity is the reduction of its phosphorylation. Tyrosinase is phosphorylated by protein kinase C-β (Park *et al.*, 1993) and is activated by the phosphorylation of serine residues in its cytoplasmic domain, which remains on the cytosolic surface of melanosomes (Park *et al.*, 1999). Therefore, inhibiting tyrosinase phosphorylation, for example by bisindolylmaleimide, a selective protein kinase C inhibitor (Park *et al.*, 2004a), reduces tyrosinase activity in cultured melanocytes as well as lightening pigmentation in the skin and hair. Further, since the second reaction in the melanogenic cascade, i.e. the conversion of L-3,4-dihydroxyphenylalanine to L-3,4-dihydroxyphenylalanine quinone, as well as other non-enzymatic reactions in the pathway, are oxidative reactions, antioxidants such as α-tocopheryl ferulate (Funasaka *et al.*, 1999; Shimizu *et al.*, 2001), magnesium L-ascorbyl-2-phosphate (Kameyama *et al.*, 1996) and 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (Kumano *et al.*, 1998), are also effective inhibitors of melanin synthesis in cultured melanoma cells.

Acceleration of tyrosinase degradation

The synthesis and the degradation of tyrosinase are tightly coupled to its function, and are influential parameters that regulate melanin synthesis. In instances where a melanogenic inhibitor decreases tyrosinase protein levels but has little effect on its mRNA levels (cf. examples below), it is like that the degradation of tyrosinase was accelerated by that agent. Early studies on the stability of tyrosinase revealed that tyrosinase is degraded endogenously in melanoma cells (Saeki and Oikawa, 1980; Jiménez *et al.*, 1988; Martínez-Liarte *et al.*, 1988). The rate of tyrosinase degradation was found to be increased by acidification of the culture medium (Saeki and Oikawa, 1980), indicating that the degradation of tyrosinase could be altered by environmental factors surrounding melanocytic cells. Recent studies of tyrosinase degradation have revealed that a variety of intrinsic factors in the epidermis or other factors have a potency to regulate tyrosinase degradation, as follows:

TGF-β1. Keratinocytes synthesize and secrete various cytokines such as IL-1α and TNF-α (Wilmer *et al.*, 1994), IL-6 (de Vos *et al.*, 1994), TGF-β1 (Sachsenmeier *et al.*, 1996), and proopiomelanocortin (POMC, the precursor for melanocyte-stimulating hormone) (Schauer *et al.*, 1994; Wintzen and Gilchrest, 1996). Among those cytokines, TGF-β1 enhanced in a dose-dependent manner the inhibition of tyrosinase and TYRP1 activities in B16 melanoma cells following treatment with cycloheximide. The mechanism proposed to explain that was a decreased half-life of tyrosinase and TYRP1, together with a decrease of tyrosinase mRNA level (Martinez-Esparza *et al.*, 1997).

TNF-α. TNF-α is a epidermal:dermal cytokine secreted in response to chemical-induced inflammation (Wilmer *et al.*, 1994), exposure to UV radiation (Kock *et al.*, 1990; de Kossodo *et al.*, 1995) and wound healing (Feiken *et al.*, 1995). TNF-α, IL-1α, IL-6, and epidermal cell-derived thymocyte activating factor have been shown to inhibit melanin synthesis in mouse melanoma cells and in normal

human melanocytes (Swope *et al*., 1989, 1991). However, the inhibitory mechanisms of those cytokines have not yet been clarified. A later study suggested that TNF-α decreases the stabilities of tyrosinase and TYRP1 (as does TGF-β1), and also reduces the levels of their corresponding mRNAs (Martinez-Esparza *et al*., 1998).

Linoleic acid. Linoleic acid is an unsaturated fatty acid (C18:2) and is a major component of biological cell membranes. Topical application of linoleic acid has been shown to decrease UV-induced hyperpigmentation of the skin (Ando *et al*., 1998; Shigeta *et al*., 2004). Fatty acids can regulate tyrosinase degradation in contrasting manners, that is, linoleic acid accelerates whereas palmitic acid (saturated fatty acid, C16:0) decelerates the degradation of tyrosinase (Ando *et al*., 1999), with little change in levels of tyrosinase mRNA (Ando *et al*., 1995). Further study showed that linoleic acid increases the amount of ubiquitylated tyrosinase which leads in turn to the accelerated degradation of tyrosinase by proteasomes (Ando *et al*., 2004).

2,2′-Dihydroxy-5,5′-dipropyl-biphenyl. 2,2′-Dihydroxy-5,5′-dipropyl-biphenyl has a phenol structure that could potentially elicit competitive inhibition of tyrosinase as does hydroquinone and arbutin, although it does not directly affect levels of tyrosinase protein or mRNA. The mechanism underlying the decreased melanin synthesis elicited by 2,2′-dihydroxy-5,5′-dipropyl biphenyl was shown to be the accelerated degradation of tyrosinase in cultured melanoma cells (Nakamura *et al*., 2003). This compound also inhibited the trafficking of tyrosinase to melanosomes due to the suppression of tyrosinase maturation.

Tetradecanoylphorbol-13-acetate/phospholipase D2. Tetradecanoylphorbol-13-acetate has been shown to decrease melanin synthesis in mouse melanoma cells via the inhibition of tyrosinase mRNA transcription (Fuller *et al*., 1990; Kuzumaki *et al*., 1993; Ando *et al*., 1995). Recently, it was also found that tetradecanoylphorbol-13-acetate activates phospholipase D2, an enzyme that hydrolyzes phosphatidylcholine to generate phosphatidic acid, and the overexpression of phospholipase D2 decreased tyrosinase levels in cultured melanoma cells. This could be blocked by proteasome inhibitors, which suggests that the tetradecanoylphorbol-13-acetate-induced downregulation of melanin synthesis is caused by a decrease in tyrosinase levels mediated by phospholipase D2-induced proteasomal degradation (Kageyama *et al*., 2004).

25-Hydroxycholesterol. 25-Hydroxycholesterol is an oxysterol that regulates cholesterol homeostasis (Schroepfer, 2000), and treatment with 25-hydroxycholesterol decreases melanin synthesis and tyrosinase activity in mouse melanocytes. However, 25-hydroxycholesterol did not directly inhibit tyrosinase activity or decrease its mRNA, but rather it enhanced the degradation of glycosylated tyrosinase after its maturation in the ER and Golgi. The accelerated degradation of tyrosinase by 25-hydroxycholesterol occurs via a proteasome-independent mechanism (Hall *et al*., 2004).

PTU. PTU is a potent inhibitor of tyrosinase (Dubois and Erway, 1946; Lerner and Fitzpatrick, 1950). Although the inhibitory effect of PTU was thought to be due to the chelation of copper atoms at the active site of tyrosinase, it was recently found that PTU also decreases the stability of tyrosinase. Metabolic labeling and immunoprecipitation analysis revealed that PTU enhances tyrosinase degradation following its maturation in the Golgi in a proteasome-independent but a endosomal/lysosomal cysteine protease-dependent manner in cultured melanocytes (Hall and Orlow, 2005). The effects of PTU on the degradation of melanogenic enzymes is similar to that of linoleic acid (Ando *et al*., 1999), that is, the effects of those agents are specific to tyrosinase and do not affect TYRP1 or dopachrome tautomerase.

Indirect regulation of tyrosinase activity

Melanin synthesis in melanocytic cells can also be modulated by the indirect regulation of tyrosinase. Epidermal pigmentation involves not only the intracellular regulation of tyrosinase activity within melanocytes but also reflects environmental influences on melanocytes, including those derived from surrounding keratinocytes. Many studies have attempted to inhibit melanin synthesis and/or tyrosinase activity indirectly by interfering with cutaneous secretory factors that can activate melanocytes. Examples include the inhibition of cell-to-cell signaling between keratinocytes and melanocytes which activates melanogenesis via paracrine cytokines such as endothelin-1, for example by a chamomilla extract (Imokawa *et al*., 1997), via the UVB-induced histamine in famotidine, an H2 receptor antagonist (Yoshida *et al*., 2002), by inhibitors of inflammation such as by glabridin found in licorice extracts (Yokota *et al*., 1998) and by *trans*-4-aminomethylcyclohexanecarboxylic acid, termed tranexamic acid (Maeda and Naganuma, 1998).

HOW THE QUALITY CONTROL OF TYROSINASE IMPACTS ITS STABILITY

Although the endogenous degradation of tyrosinase was first observed several decades ago, little had been clarified about the specific mechanism(s) that regulates tyrosinase degradation until proteasomes were found to be involved in that process (Halaban *et al*., 1997). Studies on carbohydrate modifications of tyrosinase have revealed that tyrosinase destined for degradation in the ER is proteolyzed by proteasomes via ER-associated protein degradation (ERAD) (Wang and Androlewicz, 2000; Svedine *et al*., 2004). ERAD is a mechanism for quality-control which involves retention in the ER and retro-translocation into the cytosol of misfolded or unassembled secretory proteins followed by their deglycosylation, ubiquitylation, and subsequent proteolysis by proteasomes (Bonifacino and Weissman, 1998; Plemper and Wolf, 1999; Ellgaard and Helenius, 2003). Recently, it was found that tyrosinase degradation can also occur following its complete maturation in the Golgi, which

suggests that tyrosinase is also subject to post-Golgi-associated protein degradation (Hall *et al.*, 2004; Hall and Orlow, 2005) and molecular chaperones in the ER are closely connected to tyrosinase degradation via ERAD (Ando *et al.*, 2006).

ER retention of tyrosinase in OCA followed by ERAD

OCA1 is an autosomal recessive disease caused by mutations in the gene encoding tyrosinase which result in a complete deficiency of melanin pigment in the skin, hair, and eyes (Oetting and King, 1999; Toyofuku *et al.*, 2001a). In albino melanocytes or in amelanotic melanoma cells, the aberrant retention of tyrosinase in the ER and its subsequent degradation can occur as a secondary phenomenon owing to the quality-control machinery, indicating that OCA, at least in part, is an ER retention disease (Halaban *et al.*, 1997, 2000; Berson *et al.*, 2000; Toyofuku *et al.*, 2001b). Molecular chaperones in the ER, such as calnexin and calreticulin (which assist protein maturation) play roles in the retention of misfolded proteins in the ER (Hammond and Helenius, 1995). In fact, mutations in tyrosinase or in TYRP1 enhance and prolong their association with calnexin and Bip, another molecular chaperone in the ER that recognizes hydrophobic residues of unfolded proteins before the binding of calnexin/calreticulin (Flynn *et al.*, 1991). This in turn causes the retention of those mutant proteins in the ER (Toyofuku *et al.*, 2001b) and they are subsequently degraded by ERAD coincident with their dissociation from ER chaperones (Toyofuku *et al.*, 2001a). A similar situation has also been reported for dopachrome tautomerase (Negroiu *et al.*, 2003).

The roles of oligosaccharide trimming of tyrosinase and ER molecular chaperones on tyrosinase stability

In the early stages of sugar chain processing in the ER, multiple N-linked glycans ($Glc_3Man_9GlcNAc_2$) of immature tyrosinase are trimmed sequentially by α-glucosidases I and II. The monoglucosylated *N*-glycans ($Glc_1Man_9GlcNAc_2$) of tyrosinase interact with calnexin and calreticulin and binding to those chaperones helps the folding efficiency of nascent tyrosinase (Hammond *et al.*, 1994; Hebert *et al.*, 1995; Ware *et al.*, 1995). Correctly folded tyrosinase is eventually released from the complex during the final trimming of glucose by α-glucosidase II. The CuB copper binding site is also essential, not only for the exit of tyrosinase from the ER but also for its correct maturation throughout the Golgi stack, followed by the loading of two copper atoms, which most likely occurs in the trans-Golgi network (Olivares *et al.*, 2003). This sequence is based on results showing that recombinant tyrosinase expressed in fibroblasts deficient in the Menkes copper transporter (which is localized predominantly in the trans-Golgi network), is inactive (Petris *et al.*, 2000), after which tyrosinase is further processed to its mature form (Figure 2).

The oligosaccharide trimming of sugar chains plays a pivotal role in the targeting of tyrosinase to the cytosol for degradation via ERAD (Mosse *et al.*, 2001). This is also supported by the finding that when calnexin binding to the glucosylated *N*-glycans of tyrosinase is prohibited by inhibiting α-glucosidase I, tyrosinase escapes from ERAD to melanosomes in a misfolded form that has a normal half-life (Petrescu *et al.*, 1997). Once the tyrosinase:calnexin complex has formed, inhibition of α-glucosidase II prevents tyrosinase from being released spontaneously from the complex, which results in incorrect folding and then degradation (Branza-Nichita *et al.*, 1999).

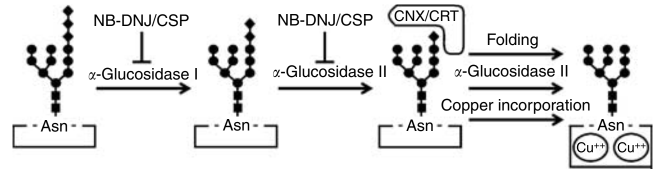

Figure 2. Simplified scheme of asparagine-linked glycan processing and maturation pathway of tyrosinase. The initial *N*-glycan of tyrosinase, $Glc_3Man_9GlcNAc_2$, is trimmed sequentially by α-glucosidases I and II in the ER. Both steps are inhibited by *N*-butyldeoxynojirimycin (NB-DNJ) and castanospermine (CSP). Nascent tyrosinase is folded by molecular chaperones, for example, calnexin (CNX) and calreticulin (CRT), through their recognition of monoglucosylated *N*-glycans of tyrosinase, followed by the acquisition of two copper atoms in the *trans*-Golgi network that gives rise to active tyrosinase. Symbols: (◆) glucose; (●) mannose; (■) *N*-acetyl glucosamine.

In contrast, the inhibition of mannose trimming of tyrosinase in the ER by mannosidase inhibitors, such as deoxymannojirimycin or kifunensine, significantly delays the rate of ERAD of tyrosinase (Wang and Androlewicz, 2000). This occurs because a mannose-trimmed isomer appears to be the substrate for the putative mannose-specific ERAD receptor, ER degradation enhancing an α-mannosidase-like protein, termed EDEM, which targets ERAD substrates for degradation (Hosokawa *et al.*, 2001). EDEM plays a role in the release of misfolded glycoproteins from the calnexin cycle (Oda *et al.*, 2003; Molinari *et al.*, 2003), and acts in concert with Yos9p, a conserved ER lectin that binds to Man8GlcNAc2 carbohydrate, to facilitate recognition of lumenal misfolded glycoproteins for ERAD (Bhamidipati *et al.*, 2005; Szathmary *et al.*, 2005; Kim *et al.*, 2005c). Yos9p also participates in a sugar-dependent recognition machinery to select misfolded substrates for ERAD (Denic *et al.*, 2006). The sum of these findings supports the role of mannose composition acting as a degradation signal.

Tyrosinase degradation by ubiquitin-proteasome dependent and independent pathways

The major pathways of protein degradation in eukaryotic cells include proteolysis in proteasomes and in lysosomes. In the quality control mechanism of proteins in the ER, the ERAD system has proven to be a fundamental axis of selective disposal of aberrant and short-lived proteins via the ubiquitin-proteasome pathway. In addition, in some instances, nascent proteins processed to post-Golgi compartments have been proposed to undergo retrograde transport back to the ER with retargeting to the ERAD pathway, or to be targeted into the endosomal system for degradation by vacuolar/lysosomal proteases (Arvan *et al.*, 2002). In fact, tyrosinase processed

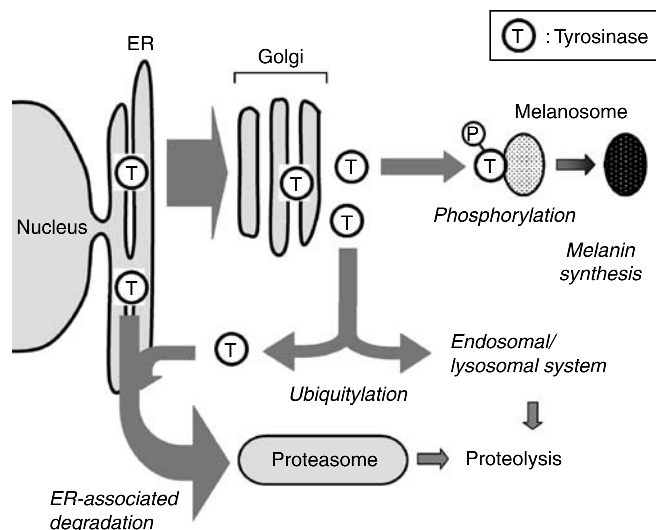

Figure 3. Schematic diagram of the tyrosinase processing and degradation pathway. After maturation in the Golgi, the trafficking of tyrosinase is mainly divided into two pathways, that is, one to melanosomes for melanin synthesis, which is regulated by the phosphorylation of the cytoplasmic domain of tyrosinase by protein kinase C-β, and the other is into the degradation machinery for proteolysis. The proteolysis of tyrosinase is also divided into two pathways, that is, one integrated into the ERAD pathway in which tyrosinase is retrotranslocated to the cytosol from the ER and degraded in proteasomes whereas the other is integrated into the endosomal/ lysosomal degradation system.

beyond the post-Golgi stage can be rapidly degraded by integration into the ERAD system, as observed in linoleic acid-treated melanoma cells (Ando *et al.*, 2006), or by the endosomal/lysosomal system, as observed in PTU-treated melanoma cells (Hall and Orlow, 2005). However, the selective mechanism(s) that determines the destination of proteins for alternative degradation at the post-Golgi stage is still uncertain (Figure 3).

Interestingly, the ERAD of tyrosinase has been proposed to imply the involvement of two distinct pathways, that is, a cytoplasmic proteasome-dependent pathway that requires association with calnexin (Liu *et al.*, 1999) and an ER-lumenal non-proteasomal pathway that does not require calnexin association. This was surmised because tyrosinase degradation can also be observed in the presence of glucosidase inhibitors that prevent the association of tyrosinase with calnexin by blocking the trimming of glucose residues down to a single residue (Wang and Androlewicz, 2000). However, the nature of the calnexin-independent pathway of tyrosinase degradation remains to be determined.

On the other hand, the detection of ubiquitylated tyrosinase with various molecular sizes has been reported (Halaban *et al.*, 1997; Mosse *et al.*, 1998; Ando *et al.*, 2004). It has been demonstrated that ubiquitylation is required for the retro-translocation of ER glycoproteins to the cytosol for degradation by proteasomes (de Virgilio *et al.*, 1998). The delivery of a retro-translocated ERAD substrate to the proteasome is catalyzed by the Cdc48-Ufd1-Npl4 complex that binds to the polyubiquitin conjugate of ERAD substrates and facilitates their presentation to proteasomes (Bays and Hampton, 2002; Ahner and Brodsky, 2004). Together with the importance of ubiquitylation in the ERAD system, it was also reported that ubiquitylation plays a role as a sorting determinant for entry into the endosomal degradative system (Arvan *et al.*, 2002). More specifically, ubiquitylation of Melan-A/MART-1, another melanosomal protein distinct from tyrosinase, has been shown to serve as a sorting signal for lysosomal degradation (Levy *et al.*, 2005). Thus, ubiquitylation plays a pivotal role not only in the ERAD system but also in the proteasome independent pathway. The intracellular location where the ubiquitylation of tyrosinase occurs remains to be determined.

Other intrinsic factors or machineries involved in tyrosinase degradation

Several studies have reported intrinsic melanogenic factors that are involved in tyrosinase stability. As one example, tyrosinase and TYRP1 bind each other and form a melanogenic protein complex; mutations in either component target the entire complex for degradation (Toyofuku *et al.*, 2001b). Mutations in TYRP1 are associated with another form of OCA, OCA type 3 (Boissy *et al.*, 1996). Mutations in TYRP1 not only accelerate its degradation, but also the degradation of normal (wild-type and functional) tyrosinase, although other melanogenic proteins (e.g. DCT and Pmel17) were not similarly affected (Kobayashi *et al.*, 1998). Tyrosinase can also be stabilized by the cofactor L-3,4-dihydroxyphenylalanine and the substrate tyrosine (Halaban *et al.*, 2001).

It has been shown that the transient aggregation of nascent proteins in the ER occurs during the initial step of interaction with molecular chaperones (Kim *et al.*, 1992; Marks *et al.*, 1995; Kellokumpu *et al.*, 1997). The oligomerization/ aggregation of tyrosinase has also been shown to be a step required for the proper maturation of tyrosinase following its chaperone-mediated folding in the ER, and that process occurs before its processing in the Golgi (Francis *et al.*, 2003). Aggregation of tyrosinase appears to represent a cellular strategy for sequestering aberrant proteins until they can be degraded i.e., the aggregates are eventually dissociated and are then targeted for degradation by proteasomes (Svedine *et al.*, 2004). Thus, aggregation of tyrosinase serves not only as an intermediate in the normal folding of tyrosinase but also as a precursor of tyrosinase degradation.

Other intrinsic factors that can modulate tyrosinase degradation include those that affect disulfide bond formation. TYRP1 possesses multiple intramolecular disulfide bonds that stabilize its structure. Treatment with dithiothreitol, a reducing agent that disrupts disulfide bridges, elicits the retention of TYRP1 in the ER by prolonging its interaction with calnexin and Bip (Negroiu *et al.*, 2000) (which affects the trafficking of glycoproteins (Machamer and Rose, 1988)) and which in turn indirectly affects tyrosinase stability.

PERSPECTIVES

It is perhaps not surprising that there are multiple approaches to controlling melanogenesis as melanin biosynthesis can be modulated by a wide variety of factors in addition to those

mentioned above. For example, melanin synthesis by melanocytic cells can be regulated by extracellular pH and also by the addition or depletion of glucose or galactose (Halaban *et al*., 2002; Watabe *et al*., 2004). Intramelanosomal pH is also an essential factor that regulates melanin synthesis (Ancans *et al*., 2001; Smith *et al*., 2004) and it has been proposed that the modulation of intracellular pH is at least in part responsible for the stability/trafficking of tyrosinase to melanosomes and thus actively regulates constitutive skin color (Fuller *et al*., 2001; Smith *et al*., 2004). Heat treatment decreases melanin synthesis via inactivation of protein phosphatase 2A (Kim *et al*., 2005b) and the trafficking or glycosylation of tyrosinase might also be affected by cholesterol homeostasis as its maturation is inhibited by cholesterol starvation (Hall *et al*., 2004). U18666A, 3β-(2-diethylaminoethoxy)-androsterone HCl and boronophenylalanine inhibit melanin synthesis via the aberrant trafficking of tyrosinase (Hall *et al*., 2003) and by inhibiting the polymerization of melanin monomers (Mishima and Kondoh, 2000), respectively. In addition, some inhibitors of melanogenesis cannot be explained by effects on tyrosinase function, for example, treatment with yohimbine, an α-2 adrenergic receptor antagonist (Fuller *et al*., 2000). One must keep in mind the model system in which effects have been shown, and that results obtained using murine melanocytes or even human melanoma cells may not be appropriate models for what occurs in human skin *in situ*.

There is no doubt that the specific inhibition of tyrosinase function would be an effective therapy to reduce skin pigmentation, although this is not easily achieved. This review reveals a potential and attractive strategy for designing novel agents that can improve skin hyperpigmentary disorders, via effects on tyrosinase. We focused on the quality control system of tyrosinase in respect to the degradation machineries such as the ERAD and the lysosomal/endosomal systems, which are affected by the process of oligosaccharide trimming, molecular chaperone engagement and protein trafficking/sorting, at least in part, via ubiquitylation. Useful agents to be selected based on those strategies not only include direct enhancers of tyrosinase degradation but also include indirect regulators of the multiple steps involved in the quality control of tyrosinase which can lead to its accelerated proteolysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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