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Effects of Captopril on Melanin Formation in B16 cells

HEUY-LING CHU[1], BOR-SEN WANG[2], LI-CHEN CHANG[3], LEE-WEN CHANG[1] AND PIN-DER DUH[1]*

[1.] Department of Food Science and Technology, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, R.O.C.
[2.] Department of applied Life Science and Healty, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, R.O.C.
[3.] Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, R.O.C.

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ABSTRACT

The effect of captopril on melanin formation in B16 cells was investigated. In B16 intracellular model system, the effect of captopril on antityrosinase activity was found dose-dependent and correlated to its ability to suppress melanin formation. The copper chelation by captopril may in part be responsible for the inhibition of tyrosinase activity. Captopril also displayed a remarkable reducing ability and significantly inhibited ROS generation. Interestingly, captopril also significantly suppressed tyrosinase mRNA expression, as determined by reverse transcription-polymerase chain reaction (RT-PCR). Overall, the results showed that the protective effect of captopril makes it a potent inhibitor of melanin formation.

Key words: captopril, antityrosinase, reactive oxygen species, melanin formation, B16 melanoma cell

INTRODUCTION

Melanins are natural pigments present in animal and plant kingdoms. Many research have been published on the melanin synthesis[1,2]. Riley[2] noted that melanin has many biological functions including antibiotic properties, strengthening element of plant cell walls, light absorption, and scavenging free radical. However, Nofsinger and others[3] reported there are also negative aspects of melanin, which play a role in generating reactive oxygen species (ROS) and in carcinogenesis. Skin exposed to ultraviolet light (UV) produces ROS; these ROS's promote melanin biosynthesis and destroy deoxyribonucleic acid (DNA) and then induce the proliferation and apoptosis of melanocytes[4,5]. Previous studies showed ROS scavengers and inhibitors of ROS production such as antioxidants may reduce hyperpigmentation or prevent new UV-induced melanogenesis[4]. Apart from the properties mentioned above, localized hyperpigmentation is a sign of abnormal pigment cell function, such as malignant melanoma[2], and overproduction of melanin causes undesirable skin coloration[6]. Melanin biosynthesis is a result of a series of oxidative reactions involving tyrosine in the presence of the enzyme tyrosinase[7]. Tyrosinase catalyzes two distinct oxidative reactions: the hydroxylation of tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to dopaquinone[8]. Quinones are highly reactive compounds and thereby polymerize to form melanins[7]. Many reports have shown melanin formation may be prevented by avoiding ultraviolet exposure, by inhibiting tyrosinase, by inhibiting melanocyte metabolism and proliferation, or by removing melanin by corneal ablation[7]. Among these, apart from avoiding ultraviolet exposure, using tyrosinase inhibitors may be a simple and effective way to prevent melanin formation[6]. Therefore, the development and searching for potential and effective tyrosinase inhibitors have received much attention.

Captopril, an angiotensin converting enzyme inhibitor, has long been used in treating congestive heart failure. In addition, captopril with a sulfhydryl functional group has been reported as a dietary thiol. Furthermore, captopril was found to be present in vegetables such as asparagus[9,10]. Regarding the biological effects of thiols, more dietary thiol supplements could regulate cell and tissue thiols in different models. Cellular thiols redox status can modulate physiological metabolism, including its immune regulating and anti-inflammatory properties, scavenging reactive oxygen species, antioxidant properties, antitumor, antifibrotic and cytoprotective effects[11-14]. As to melanin biosynthesis, captopril has been demonstrated to inhibit tyrosinase activity because of its binding the enzyme at its active site[15]. However, the mechanism of anti-tyrosinase activity of captopril is not yet understood although captopril has been described as a tyrosinase inhibitor. Thus, the present study was aimed at exploring the effect of captopril against melanin formation in B16 cells, and the mechanism of action also evaluated.

* Author for correspondence. Tel: +886-6-2668618;
Fax: +886-6-2668618; E-mail: ipdduh@mail.chna.edu.tw

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MATERIALS AND METHODS

I. *Reagents*

Captopril, 2',7'-dichlorofluorescin diacetate (DCFH-DA), synthetic melanin, dimethyl sulfoxide (DMSO), and mushroom tyrosinase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). L-3,4-dihydroxyphenylalanin (L-DOPA) was obtained from Acros Organic (Geel, Belgium). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from E. Merck (Darmstadt, Germany). Trizol reagent and RT-PCR kit were obtained from Invitrogen (Carlsbad, CA, USA).

II. *Determination of Reducing Activity*

The reducing power of captopril was determined as previously described[16]. Captopril in phosphate buffer (2.5 mL, 0.2 M, pH 6.6) was added to potassium ferricyanide (2.5 mL, 10 mg/mL), and the mixture was incubated at 50°C for 20 min. TCA (2.5 mL, 100 mg/mL) was added to the mixture, which was then centrifuged at 650 g for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), and then the absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

III. *Determination of Copper Chelating in Tyrosinase*

Copper chelating in tyrosinase was determined as previously described[17] with slight modification. The mixture consisting of 0.1 mL test compound (0.1 mM), 0.7 mL 10 mM phosphate buffer (pH 6.8), and 0.2 mL mushroom tyrosinase (250 U/mL), was incubated at 25°C for 30 min followed by the UV-visible spectra (200-500 nm) acquisition.

IV. *Cell Culture*

B16 mouse melanoma cells were cultured in 9 mL DMEM medium containing 1 mL heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2/95% air controlled incubator[18].

V. *Cell Viability Assay*

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide) assay was performed as previously described[19]. MTT is a tetrazolium salt and can be converted to insoluble formazan by mitochondrial dehydrogenase in living cells. Briefly, cells were dispensed into 96-well plates and captopril was added and cultured for 24 h. Then, 20 μL MTT (5 mg/mL stock solution) were added to each well. After 1 h, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of DMSO. The optical density of each well was measured on an Anthos 2010 microplate reader at 570 nm.

VI. *Evaluation of ROS in B16 Cells*

To assess the generation of ROS in B16 cells, DCFH-DA was introduced to penetrate the cell membranes and hydrolyzed by intracellular esterase to form dichlorofluorescin (DCFH)[18]. Subsequently, DCFH reacted with ROS generated by intracellular stress to produce highly fluorescent DCF which emitted fluorescence when excited at 485 nm. Various concentrations of captopril in DMSO were incubated at 25°C for 30 min in the presence of DCFH-DA (50 μM). After incubation, ROS generation was detected on a Bio-Tek FLx800 microplate fluorescence reader with excitation and emission wavelengths of 485 and 530 nm, respectively. Inhibition (%) = (1 – B / A) × 100%; A: value of fluorescence without sample, and B: value of fluorescence with sample.

VII. *Assay of Inhibitory Tyrosinase Activity*

The anti-tyrosinase activity was determined as the degree of inhibition on tyrosinase-catalyzed oxidation of L-DOPA as previously described[18]. After B16 cells were cultured with or without captopril (0-400 μg/mL) for 24 h, they were washed with ice-cold PBS, and then treated with 1 mL lysis buffer [PBS containing 1% Nonidet P-40, 1% SDS and 0.2% proteinase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. Cellular lysates were centrifuged at 10,000 g at 4°C for 20 min. The supernatants were collected and the protein contents were determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and the remaining was stored at -80°C for the subsequent assays of anti-tyrosinase activities. The reaction mixture consisted cell extract supernatant (0.1 mL), 25 mM phosphate buffer (0.8 mL, pH 6.8), and L-DOPA (3.8 mM**)** was added in to read the absorbance at 475 nm for 30 min. The reaction was performed at 25°C. The value in the absence of captopril was used as the control.

$$\text{Inhibition (\%)} = [1 - (OD_{475} \text{ in sample} / OD_{475} \text{ in control})] \times 100\%$$

VIII. *Melanin Assay*

Melanin assay was performed using procedures described previously[18] with slight modifications. Test samples were added to the cell and cultured for 24 h. After washing two times in PBS, cells were harvested by short incubation with trypsin-EDTA. An aliquot was used for cell counting and the remaining cells were centrifuged at 10,000 g for 15 min. The cells were lysed in 500 μL of 5 N NaOH at 100°C for 1 h, and 200 μL portions of crude cell extract were transferred to 96-well plates. Melanin concentrations were calculated by comparison of the absorbance at 405 nm of unknown samples with a standard curve obtained using synthetic melanin (0-200 μg/mL).

IX. *Reverse Transcriptase-Polymerase Chain Reaction*

Total RNA was isolated from cells by using Trizol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. Then, RNA concentrations were determined spectrophotometrically and 1 μg RNA was converted to the first strand cDNA and amplification of target cDNA using an Invitrogen SuperScript TM III First-strand synthesis system. The same amount of the resulting cDNA was then used for amplification using the following primers: tyrosinase forward 5′-GGCCAGCTTTCAGGCAGAGGT; and downstream 5′-TGGTGCTTCATGGGCAAAATC. The cDNA sequence of GAPDH was also amplified as control in a similar way using primer pair 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. A thermal cycle of 60 sec at 94°C, 60 sec at 56°C and 60 sec at 72°C was carried for 25 cycles. PCR products were analyzed on 1.2% agarose gels and amplified cDNA bands were detected by ethidium bromide staining[20].

X. *Statistical Analysis*

All data were recorded as means ± SD. Statistical analysis involved the use of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Previous studies showed *p*-coumaric acid not only was a natural dietary antioxidant playing an important role against oxidative damage in corn bran[21], but also a strong tyrosinase inhibitor displaying competitive inhibition to eggplant tyrosinase[22]. It could be an antimelanogenesis agent in B16 cells[23]. According to the facts mentioned above, *p*-coumaric acid was used as a reference compound in this study.

I. *Effect of Captopril on Cell Viability in B16 Cells*

To examine the biological action of captopril in cellular system, B16 cells were used as an *in vitro* model. Before *in vitro* studies were performed, the effect of captopril on cell viability was determined. As assayed with MTT method, cell viability of the B16 cells treated with captopril in the range of 0-400 μg/mL was > 95% (Table 1), indicating that captopril exerted no cytotoxicity to B16 cells.

II. *Effect of Captopril on ROS Generation in B16 Cells*

Under physiological metabolism, the excessive level of ROS could decrease the intracellular antioxidant capacity and thus produce oxidative stress. In addition, ROS may induce and enhance the development of melanin formation[18]. Therefore, removing ROS may contribute to the inhibition of melanin formation. Thus, in the present investigation, the intracellular level of ROS was measured using a fluorescent probe DCFH-DA by fluorospectrometry. As shown in Table 2, the fluorescence intensity decreased as compared to control when captopril was added to B16 cells, indicating the inhibition of ROS generation by the treatment of captopril. Wang *et al*.[6] noted the antioxidants may prevent or delay pigmentation *via* various routes, including by scavenging ROS and RNS (reactive nitrogen species), or by reducing *o*-quinones or other intermediates in melanin biosynthesis, consequently, which in turn slows down the pigmentation. Without thio-containing compounds, dopaquinone spontaneously converts initially to dopochrome and then to indole-5, 6-quinone or indole-5, 6-quinone 2-carboxylic acid. After multi-biosynthesis steps, further polymerization yields melanin[8]. In addition, 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, which are melanin precursors in melanogenesis, can be converted into the corresponding melanin pigments by the action of lipoxygenase/H_2O_2 system or due to the generation of ROS, revealing ROS generation may heighten melanin formation[24]. These observations imply a strong relationship between ROS generation and melanin formation. In other words, the suppression of ROS by captopril is in part a contributor to the inhibition of melanin formation.

III. *Effect of Captopril on Reducing Activity*

Figure 1 showed the reducing ability of captopril. The reducing ability of captopril increased with the concentration of captopril. The equation of reducing ability (Y) and concentration of captopril (X) used is $Y = 0.096\ X + 0.00384$ ($r^2 = 0.972$, $p < 0.05$), indicating the reducing ability correlated well with concentration of captopril. At a concentration of 100 μg/mL, the reducing ability of captopril was inferior to that of ascorbic acid which is a strong reducing agent, but superior to that of *p*-coumaric acid. Reducing ability is related to a reducing agent that contributes to the breaking of

Table 1. Effect of captopril on B16 melanoma cell viability for 48 h

Concentration (μg/mL)	Cell viability (% of control)
100	102.2 ± 1.8^a
200	98.8 ± 3.4^a
400	101.7 ± 4.2^a

Data are presented by means ± SD (n = 3). Values with the same superscripts (a) in each column are not significantly different ($p < 0.05$).

Table 2. The effect of captopril on ROS formation in B16 cells by using the DCFH-DA fluorescence probe

Compound	Concentration (μg/mL)	Inhibition of ROS generation (% of control)
Captopril	100	17.1 ± 2.3^a
	200	37.4 ± 2.0^b
	400	42.5 ± 3.2^b

The data were displayed with mean ± SD for (n = 3). Mean values with different superscripts (a,b) in each column are significantly different ($p < 0.05$).

a radical chain by donating a hydrogen atom[25]. Moridani[26] noted that ascorbic acid prevented the *o*-quinone formation when 4-hydroxyanisole was metabolized by tyrosinase/O_2. In addition, SH-containing compounds are effective inhibitors of pigment formation[27]. In the current study, captopril exerted significant reducing action (Figure 1) and thereby reduced the product dopaquinone back to L-DOPA. Thus, the reducing ability may partly contribute to inhibit melanin formation by donating a hydrogen atom from the SH group in captopril.

IV. *Effect of Captopril on Tyrosinase and Melanin in B16 Cells*

Table 3 shows the effect of captopril on tyrosinase activity and melanin content of B16 cell. The inhibition of tyrosinase activity increased with the increasing concentration of captopril. Also, the inhibitory action on melanin formation by captopril occurred in a concentration dependent manner. Apparently, inhibition of melanin synthesis was related to the level of tyrosinase inhibition. Melanin is synthesized by a multi-step pathway, and apart from tyrosinase, synthesis is also controlled by other enzymes such as 5,6-dihydroxyindole-2-carboxylic acid oxidase and dopachrome tautomerase[28]. However, tyrosinase plays a crucial role in melanin biosynthesis and accounts for melanization in animals and browning in plants[29]. Therefore, the inhibition of tyrosinase activity by captopril may contribute to the reduction of melanin formation.

V. *Effect of Captopril on Copper Chelating*

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monophenolase and as an *o*-diphenolase[30]. Therefore, copper chelation may contribute to the inhibition of tyrosinase activity. To see whether captopril can chelate copper in the enzyme, the captopril was let to react with tyrosinase and the wavelength shift was monitored for this aim[17]. Based on the data gained from Figure 2, the absorption maximum of captopril (in profile a) shifted to short wavelength after it reacted with tyrosinase (profile b). This observation agreed with the report of Kubo and Kinst-Hori[17] who noted that kaempferol made the noticeable shift to a short wavelength as it chelated copper in the tyrosinase. This finding suggested captopril be a copper chelator[15,17], and this copper chelation mechanism may account for its inhibition of tyrosinase activity.

Table 3. The effects of captopril on tyrosinase activity and melanin formation in B16 cells

Captopril concentration (μg/mL)	Tyrosinase activity inhibition(%)	Melanin formation inhibition(%)
100	22.5 ± 2.8^a	15.1 ± 2.9^a
200	27.3 ± 1.6^b	19.6 ± 2.6^a
400	35.1 ± 2.5^c	26.5 ± 2.1^b

The data were displayed with mean ± SD for n = 3. Values with different superscripts (a,b,c) in a column are significantly different ($p < 0.05$).

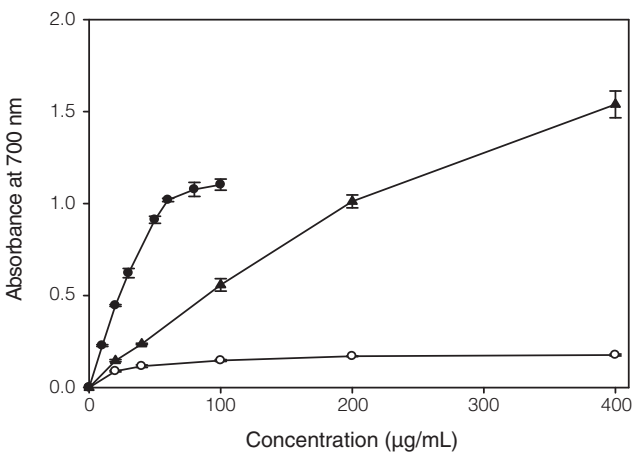

Figure 1. The reducing ability of captopril (▲), *p*-coumaric acid (○) and ascorbate (●). The reducing power of captopril was determined as described in Methods. The absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. Results are means ± SD for n = 3.

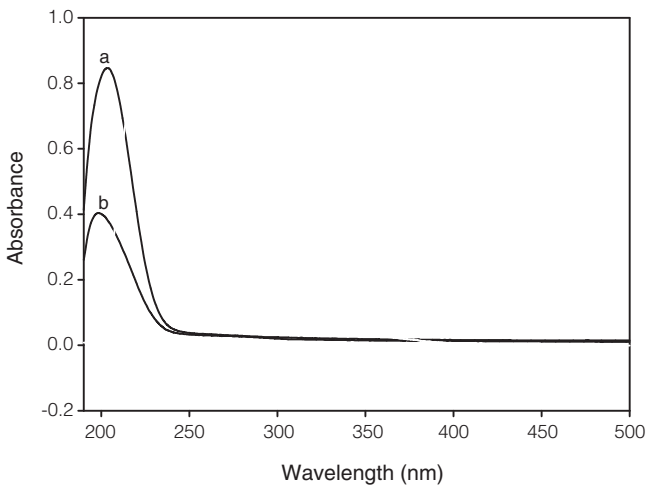

Figure 2. Spectral shift of captopril (0.1 mM): (a) without and (b) with 50 units tyrosinase. The UV-visible spectras (200-500 nm) were determined as described in Methods.

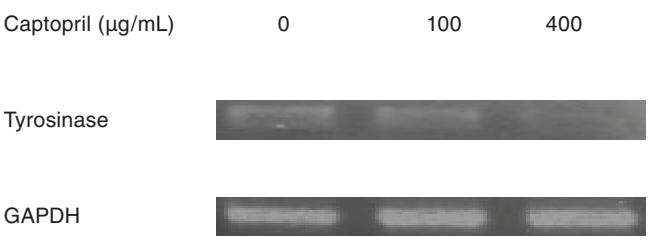

Figure 3. Effect of captopril on tyrosinase mRNA production in B16 cells. After 24 h of treatment, mRNA of tyrosinase and GAPDH in cells were analyzed by the protocol described in Methods.

VI. *Effect of Captopril on mRNA Level of Tyrosinase in B16 Cells*

To further explain a possible mechanism responsible for the lack of tyrosinase activity in B16 cells, the transcription of tyrosinase gene was further clarified. Thus, the expressions of tyrosinase mRNA were examined by RT-PCR analysis. The effects of captopril on the expression of tyrosinase mRNA in B16 cells is shown in Figure 3. The gene for GAPDH served as the housekeeping gene. The levels of mRNA encoding tyrosinase were found to be down-regulated by captopril at the concentration range of 100 to 400 μg/mL. Apparently, the presence of captopril in B16 cells suppressed the expression of tyrosinase mRNA. This observation may also be responsible for the antityrosinase activity and reduction of melanin in B16 cells.

In summary, captopril inhibited tyrosinase activity thereby reducing the melanin biosynthesis. This effect may be chiefly because of its reducing ability, chelating copper and the inhibition of tyrosinase expression. In addition, inhibition of ROS formation by captopril may be one of the contributors to the reduction of melanin formation.

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