Acta Derm Venereol (Stockh) 1983; 63: 463–467

Protein-bound Dopa and 5-S-Cysteinyldopa in Non-melanogenic Tissues

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Ito S, Jimbow K, Kato T, Kiyota M, Fujita K. Protein-bound dopa and 5-S-cysteinyldopa in non-melanogenic tissues. Acta Derm Venereol (Stockh) 1983; 63: 463–467.

To explore the possibility that dopa and 5-S-cysteinyldopa, precursors of melanin, can be produced in non-melanogenic tissues, this study examined the contents of the free and protein-bound forms of two catechols in non-melanogenic tissues of mice and rats, and compared the urinary excretion of free catechols in black and albino mice. Considerable amounts of protein bound dopa and 5-S-cysteinyldopa were present in the hair of tyrosinase-negative, albino mice and white mice, the latter completely lacking follicular melanocytes. The liver, kidney and brain of mice and the adrenals of rats also contained small amounts of these catechols. Both black and albino mice excreted free dopa and 5-S-cysteinyldopa, the amounts of which did not differ significantly in the two animals. It is suggested that oxidation mechanism(s) other than tyrosinase may participate in the synthesis of these catechols in proteins. Turnover of 5-S-cysteinyldopa-containing proteins may lead to the release of this catechol into blood and eventually to excretion into urine. *Key words: Dopa; Cysteinyldopa; Melanin; Melanogenesis; Tyrosinase; Melanoma.* (Received March 28, 1983.)

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Pigmented tissues such as black and red hair, as also malignant melanoma, contain dopa and cysteinyldopas. 5-S-Cysteinyldopa, a major precursor of pheomelanin, is present in appreciably larger quantities in red-haired skin than in black-haired skin of guinea pigs (6). In humans, 5-S-cysteinyldopa is excreted into urine after UV exposure (15). These two forms of catechol are always found in the hydrolysates of human and animal melanomas (1, 16). It has been suggested that the amounts of urinary excretion of 5-S-cysteinyldopa may parallel the stages of melanoma patients, thus making it a useful tool for estimating the degree of melanoma invasion (2).

We have recently found dopa and 5-S-cysteinyldopa in the hydrolysates of black and yellow mouse hair as well as of B16 and Harding Passey mouse melanomas (7). Active participation of tyrosinase is suggested for the synthesis of these protein-bound catechols (1). However, biological systems other than tyrosinase can also mediate the hydroxylation of phenolic moiety and the further oxidation of catecholic moiety. If the oxidation takes place on tyrosine residues in proteins, protein-bound dopa and cysteinyldopas may be formed (1). This study examined (*a*) whether protein-bound dopa and 5-S-cysteinyldopa are also present in non-melanogenic tissues, and (*b*) whether these melanin precursors are excreted into the urine of albino animals in which active tyrosinase is absent and no melanin synthesis occurs.

MATERIALS AND METHODS

Non-melanogenic organs of liver, kidney, brain and adrenal were obtained from black (C57BL) and albino (A/J, ICR) mice, and from albino rats (Wistar) following cervical dislocation. Hair follicles

Table I. *Free and protein-bound dopa and 5-S-cysteinyldopa in non-melanogenic tissues and melanoma*

Tissue	Animal (strain)	n[a]	ng/100 mg tissue[b] (μmol/mol tyrosine)[c]		
			Free		Protein-bound
			Dopa	5-S-CD[d]	Dopa
Hair	Mouse (A/J)	3	24±23	8±3	7 000±1 500 (1 300±230)
	Mouse (ICR)	3	21±10	14±20	2 600±260 (470±40)
	Mouse (C3H, mi^{bw}/mi^{bw})[e,f]	1	12	<1	5 100 (1 000)
Liver	Mouse (C57BL, A/J, ICR)	3	3±1	2±1	200±15 (240±31)
Kidney	Mouse (C57BL, A/J, ICR)	3	5±2	5±2	130±15 (170±35)
Brain	Mouse (ICR)	2	4±1	0.2	46±14 (110±42)
Adrenal	Rat (Wistar)	7[g]	150	9	220 (470)
Melanoma[h]	Mouse (ICR)	3	850±430	880±130	3 200±400 (6 400±920)

[a] Number (n) of animals.
[b] Mean ± standard deviation.
[c] Values in parentheses represent μmol of catechol per 1 mol of tyrosine in the hydrolysate.
[d] 5-S-Cysteinyldopa.
[e] Examined by incubating the plucked hair bulb in tyrosine solution with a control of C57 black hair.
[f] This strain of mouse lacks follicular melanocytes. Gift from Dr W. C. Quevedo Jr, Brown University, USA.
[g] One determination was made on adrenals from 7 rats.
[h] Data of this part were taken from our previous report (7).
[i] Mean of two experiments expressed as unit/mg protein.

were plucked from the dorsal skin of black (C57BL), albino (A/J, ICR) and white (C3H) mice. Details of the procedures for the determination of protein-bound dopa and 5-S-cysteinyldopa followed our previously described method (7). Briefly, tissue was homogenized in 0.4 M $HClO_4$ and, after centrifugation, the precipitate was hydrolysed by refluxing in 6 N HCl containing 0.5% cysteamine hydrochloride. Catechols in the extract and hydrolysate were adsorbed on alumina at pH 8.6, eluted with 0.4 M $HClO_4$, and then determined by high-performance liquid chromatography (HPLC) with electrochemical detection. Tyrosine in the hydrolysate was also determined by HPLC (7).

Tyrosinase activity of plucked hair follicles was determined by immersing the hair bulbs (anagen

Table II. *Formation of dopa and 5-S-cysteinyldopa from tyrosine and cysteine catalysed by* Fe^{2+}

Reactants[a]	Products (μmol/mol tyrosine)[b]	
	Dopa	5-S-Cysteinyldopa
Tyrosine 5 mg[c] + cysteine 5 mg	17±8	ca 0.3
Tyrosine 5 mg + cysteine 5 mg + Fe^{2+} 0.05 mg[d]	53±26	ca 0.5
Tyrosine 5 mg + cysteine 5 mg + tryptophan 5 mg + Fe^{2+} 0.05 mg	29±9	ca 0.5

[a] A mixture of the reactants in 10 ml of 6 N HCl containing 0.5% cysteamine hydrochloride was heated under reflux for 24 h and treated as described (7).
[b] Mean ± standard deviation for 3 experiments. Values represent μmol of catechol per 1 mol of tyrosine in the hydrolysate.
[c] This amount is an average value for those in tissue samples.
[d] This amount is similar to or above those in tissue samples.

5-S-CD	Tyrosinase (dopa-oxidase) activity
1 600±110 (170±42)	0.00[e]
690±270 (95±55)	0.00
1 600 (180)	–
10±3 (7±3)	0.00 (0.07)[i]
11±1 (9±3)	0.00 (0.17)
4±1 (5±1)	0.00 (0.00)
11 (15)	0.00 (0.00)
880±210 (1 100±360)	2.91 (5.29)

phase) in 0.5 mM tyrosine, 50 mM phosphate buffer solution (pH 6.8) with addition of a catalytic amount of dopa (0.025 mM) for 4 h at 37°C. For the determination of tyrosinase activity in non-melanized organs, tissue was minced with scissors, washed with phosphate-buffered saline, homogenized in phosphate buffer (pH 6.8, 50 mM), followed by centrifugation at 105.000 g for 30 min. The particulate (membrane-bound) form of tyrosinase was released by treating the pellet with 0.1% Brij 35 made up in 50 mM phosphate buffer (pH 6.8). Brij treatment was carried out with a Polytron homogenizer (Kinematika, Switzerland), gage 5, for 30 sec, at room temperature. Tyrosinase activity of the solubilized, particulate fraction was determined by incubating the samples in 0.5 mM tyrosine plus a catalytic amount of dopa (0.025 mM) made up in 50 mM phosphate buffer (pH 6.8) at 37°C for 1 h. Dopa-oxidase activity was measured by incubating the particulate fractions in 3 mM dopa made up in 50 mM phosphate buffer, pH 6.8, for 10 min at 37°C. The enzyme unit of tyrosinase and dopa oxidase was calculated by measuring absorbancy at 475 nm with extinction coefficiency of 3 600, following our previously described method (13). Harding Passey mouse melanoma was used as a control. Protein concentration was determined by the method of Lowry et al. (14).

To determine the level of free dopa and 5-S-cysteinyldopa excreted in urine, 24-h urine was collected from black (C57BL) and albino (A/J) mice. Five animals of each species were housed separately in metabolic cages and urine was collected in a beaker containing 0.5 ml of 0.5 M HCl.

RESULTS

Table I indicates the presence of considerable amounts of protein-bound dopa and 5-S-cysteinyldopa in the plucked hair of non-pigmented albino (A/J and ICR) and white (C3H) mice, the latter lacking follicular melanocytes. Tyrosine test on plucked hair follicles indicated that none of the active tyrosinase was present in hair of A/J and ICR albino mice. The liver, kidney and brain of the mice and the adrenals of the rats also contained small amounts of dopa and 5-S-cysteinyldopa in the bound form (Table I). Again, these non-melanogenic organs did not reveal any tyrosinase activity, though liver and kidney showed a trace of dopa-oxidase activity which was quite low, compared with that of control, melanoma tissue. Although the concentrations of the protein-bound catechols in normal tissues in terms of ng/100 mg tissue were less than one-tenth of those in amelanotic hair, the difference was much smaller when compared in terms of μmol/mol of tyrosine residues. The level of bound dopa in the adrenal was not significantly higher than that in other normal tissues, indicating that tyrosine hydroxylase, a unique enzyme in the adrenal, is not capable of hydroxylating tyrosine residues in proteins. Free dopa and 5-S-cysteinyldopa levels in the non-melanogenic tissues were much lower than those of the bound form

(Table I). The relatively high level of free 5-S-cysteinyldopa was noted in the kidney, similar to the report of Agrup et al. (3). The high level of free dopa and 5-S-cysteinyldopa in the adrenal may be related to the fact that it is a catecholamine-producing organ.

Urinary excretion of free dopa and 5-S-cysteinyldopa was compared between C57 black and A/J albino mice. Interestingly, there was no significant difference in urinary excretion (24 h) of free dopa and 5-S-cysteinyldopa between A/J albino and C57 black mice. Urinary dopa was 43±24 ng (mean ± standard deviation) in C57 black mice (n=5) and 37±25 ng in A/J albino mice (n=5), while urinary cysteinyldopa was 52±11 ng in C57 black mice and 44±14 ng in A/J albino mice.

There was a possibility that dopa and 5-S-cysteinyldopa could be produced artificially from tyrosine and cysteine under the hydrolytic conditions. Iron ions are known to catalyse the hydroxylation of aromatic compounds (17). Therefore, this possibility was examined and the results are summarized in Table II. Although these catechols were in fact produced by the Fe^{2+}-catalysed oxidation from tyrosine and cysteine, the yields were much lower than the tissue levels. Furthermore, other tissue constituents, such as tryptophan, appeared to have some preventive effect on the hydroxylation of tyrosine, possibly by competing for hydroxylating species.

DISCUSSION

High levels of protein-bound dopa and 5-S-cysteinyldopa were noted in pigmented hair and melanoma (7). It has been suggested that tyrosinase is the major factor catalysing the synthesis of bound dopa and 5-S-cysteinyldopa in pigmented tissues. In this study, however, protein-bound dopa and 5-S-cysteinyldopa were also found in non-melanogenic tissues, including those normal tissues in which melanocytes and tyrosinase are absent, e.g., hair of C3H white mice, liver, spleen, brain and adrenal. Thus, it is indicated that oxidation mechanism(s) other than tyrosinase may participate in the synthesis of these catechols in proteins. The hydroxyl radical is known to bring about the hydroxylation of aromatic compounds (17). We have previously shown that the peroxidase–H_2O_2 system (8), oxygen radicals, superoxide and hydroxyl radicals (9, 10, 11) can mediate the formation of cysteinyldopas from dopa and cysteine. These active oxygens are produced in many biological processes (4).

It is generally accepted that cysteinyldopas are formed only in active melanocytes and therefore the urinary level of 5-S-cysteinyldopa reflects well the progression of melanoma (2). Recently, the presence of free 5-S-cysteinyldopa was reported in the spleen and heart of guinea pigs, suggesting the presence of a specific catechol-containing structure in the non-melanogenic organs (3). Our findings of bound 5-S-cysteinyldopa in normal non-melanogenic tissues as well as free 5-S-cysteinyldopa in the urine of tyrosinase-negative, albino mice indicate that excretion of urinary 5-S-cysteinyldopa by mechanism(s) other than the tyrosinase-mediated reaction may exist. Dopa and cysteine in either free or bound form are necessary for the production of 5-S-cysteinyldopa in vivo. However, free dopa is hardly available in large amounts in non-melanogenic tissues. Interestingly, 5-S-glutathionedopa is formed in the spleen of albino rats only after dopa injection (5).

By contrast, the relatively high level of protein-bound dopa in normal tissues could favor the 5-S-cysteinyldopa production. Turnover of 5-S-cysteinyldopa-containing proteins may lead to the release of this catechol into blood and eventually to excretion into urine, inasmuch as there are no significant amounts of free dopa and 5-S-cysteinyldopa in the tissues and 5-S-cysteinyldopa is not rapidly metabolized as compared with L-dopa (12). Furthermore, wide ranges of urinary excretion of free 5-S-cysteinyldopa in melanoma

patients as well as normal subjects (2) may partly be related to the production of bound dopa and 5-S-cysteinyldopa in the non-melanogenic tissues where catalytic mechanism(s) other than tyrosinase may be involved.

ACKNOWLEDGEMENT

This study was supported by Grant-in-Aid for Cancer Research (no. 58–42) (K. J.) from the Ministry of Health and Welfare, Japan, and (no. 58010030. 58390016. 58480265) from the Ministry of Education, Science and Culture, Japan. We are grateful to Miss Tazuko Niikawa for her secretarial help.

REFERENCES

1. Agrup G, Hansson C, Rorsman H, Rosengren AM, Rosengren E. Free and bound 5-S-cysteinyldopa and dopa in human malignant melanomas. Acta Derm Venereol (Stockh) 1978; 58: 270.
2. Agrup G, Agrup P, Andersson T, Hafström L, Hansson C, Jacobsson S, Jönsson P-E, Rorsman H, Rosengren A-M, Rosengren E. Five years' experience of 5-S-cysteinyldopa in melanoma diagnosis. Acta Derm Venereol (Stockh) 1979; 59: 381.
3. Agrup G, Hansson C, Rorsman H, Rosengren E. 5-S-Cysteinyldopa, dopa, and dopamine in the kidney and some other tissues. Acta Derm Venereol (Stockh) 1980; 60: 63.
4. Bors W, Saran M, Czapski G. The nature of intermediates during biological oxygen activation. 1980; In: Bannister WH, Bannister JV, eds. Biological and clinical aspects of superoxide and superoxide dismutase. Amsterdam: Elsevier, 1.
5. Fehling C, Hansson C, Poulsen J, Rorsman H, Rosengren E. Formation of glutathionedopa in albino rats after dopa injection. Acta Derm Venereol (Stockh) 1981; 61: 339.
6. Hansson C, Agrup G, Rosman H, Rosengren E. Dopa and 5-S-cysteinyldopa in the serum of albino, black, and red guinea pigs. Acta Derm Venereol (Stockh) 1980; 60: 155.
7. Ito S, Homma K, Kiyota M, Fujita K, Jimbow K. Characterization of structural properties for morphologic differentiation of melanosomes. III. Free and protein-bound dopa and 5-S-cysteinyldopa in B16 and Harding-Passey melanomas. J Invest Dermatol 1983; 80: 207.
8. Ito S, Fujita K. Formation of cysteine conjugates from dihydroxyphenylalanine and its S-cysteinyl derivatives by peroxidase-catalyzed oxidation. Biochim Biophys Acta 1981; 672: 151.
9. Ito S, Fujita K. New possible routes for the biosynthesis of cysteinyldopas and related metabolites. In: Seiji M, ed. Pigment Cell 1981; Univ. of Tokyo Press, Tokyo: 1981: 85.
10. Ito S, Fujita K. Conjugation of dopa and 5-S-cysteinyldopa with cysteine mediated by superoxide radical. Biochem Pharmacol 1982; 31: 2887.
11. Ito S. One-step synthesis of (2-amino-2-carboxyethylthio)dopas (cys-dopas) from dopa and cysteine by hydrogen peroxide in the presence of iron–EDTA complex. Bull Chem Soc Jpn 1983; 56: 365.
12. Ito S, Teradaira R, Fujita K. Distribution and metabolism of tritium-labelled 5-S-cysteinyldopa in mice. Biochem Pharmacol 1980; 29: 3277.
13. Jimbow K, Jimbow M, Chiba M. Characterization of structural properties for morphological differentiation of melanosomes. I. Purification of tyrosinase by tyrosine affinity chromatography and its characterization in B16 and Harding Passey melanomas. J Invest Dermatol 1981; 77: 213.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265.
15. Rorsman H, Agrup G, Falck B, Rosengren A-M, Rosengren E. Exposure to sunlight and urinary excretion of 5-S-cysteinyldopa. In: Riley V, ed. Pigment cell, vol. 2 Basel: S. Karger, 1976: 284.
16. Takahashi H, Fitzpatrick TB. Large amounts of dioxyphenylalanine in the hydrolysate of melanosomes from Harding Passey mouse melanoma. Nature 1966; 209: 888.
17. Willson RL. Hydroxyl radicals and biological damage in vitro: what relevance in vivo? In: Oxygen free radicals and tissue damage, Ciba Foundation Series 65. Amsterdam: Excerpta Medica, 1979: 19.