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CONJUGATION OF DOPA AND 5-*S*-CYSTEINYLDOPA WITH CYSTEINE MEDIATED BY SUPEROXIDE RADICAL

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Abstract—Cytotoxicity of catechols has been ascribed to their binding with proteins through sulfhydryl groups. Superoxide radical (O_2^-) generated in hypoxanthine–xanthine oxidase system at pH 7.4 mediated conjugation of dopa with cysteine to form cysteinyldopas. Similarly, 5-*S*-cysteinyldopa gave 2,5-*S*,*S*-dicysteinyldopa. The rates of oxidation of the catechols by O_2^- appear to be comparable to that of reduction of nitro-blue tetrazolium by O_2^-. These results suggest that catechols may exert cytotoxicity in cells where biochemical defence against O_2^- or the quinone oxidation products is not sufficient.

Catechols exert cytotoxicity in a variety of biological systems. Examples include (1) the selective toxicity of 3,4-dihydroxyphenylalanine (dopa) and its analogues to tumour cells [1], (2) the hepatic injury by α-methyldopa [2], and (3) the destruction of catecholamine neurons by 6-hydroxydopamine [3]. Two possible mechanisms for the cytotoxic actions of catechols have been proposed [4]: (1) the quinone oxidation products of catechols, being highly electrophilic, bind covalently with proteins through nucleophilic sulfhydryl groups [5]; or (2) autoxidation of labile catechols produces cytotoxic superoxide and hydroxyl radicals and hydrogen peroxide.

The catechol 5-*S*-cysteinyldopa arises by the addition of cysteine to dopaquinone produced oxidatively from dopa [6]. This unique amino acid is excreted in high levels in the urine of patients with melanoma metastases [7]. Recently, we found that 5-*S*-cysteinyldopa is toxic to a variety of human tumour cell lines in culture and possesses antitumour activity against murine L1210 leukaemia and B-16 melanoma [8].

We have shown that oxidation of dopa by mushroom tyrosinase in the presence of cysteine gives 5-*S*-cysteinyldopa, 2-*S*-cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa in a ratio of 15:3:1 [9]. Peroxidase can also catalyze the conjugation of dopa and cysteinyldopas with cysteine [10]. In our continuing efforts to elucidate the mechanism of cytotoxic action of catechols [11], we have examined whether superoxide radicals (O_2^-) can mediate the conjugation of dopa and 5-*S*-cysteinyldopa with cysteine.

MATERIALS AND METHODS

Xanthine oxidase and catalase were purchased from Boehringer Mannheim GmbH, West Germany, and the former was dialyzed against 0.05 M potassium phosphate buffer (pH 7.4) before use. Superoxide dismutase, cytochrome *c*, and nitro-blue tetrazolium were obtained from Sigma Chemical Co., St. Louis, MO. 5-*S*-Cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were chemically prepared by us [11].

Reductions of cytochrome *c* and nitro-blue tetrazolium by a hypoxanthine–xanthine oxidase system at pH 7.4 were followed at 550 nm [12] and 560 nm [13], respectively.

RESULTS

O_2^--Mediated conjugation of dopa and 5-S-cysteinyldopa with cysteine. Xanthine oxidase produces O_2^- when acting on xanthine or hypoxanthine. To minimize the autoxidation of the catechols and cysteine, we used relatively large amounts of xanthine oxidase and a short reaction time of 5 min. Table 1 shows that the O_2^- generated at pH 7.4 could mediate the conjugation of dopa with cysteine to give 5-*S*-cysteinyldopa. 2-*S*-Cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were also produced in smaller amounts [9]. The production of cystine was negligible. Reaction of 5-*S*-cysteinyldopa with O_2^- in the presence of cysteine also gave the product of conjugation, 2,5-*S*,*S*-dicysteinyldopa (Table 1).

As the present hypoxanthine–xanthine oxidase system generated 116 μM O_2^- in the first 5 min, and two moles of O_2^- are required for the oxidation of 1 mole of dopa to dopaquinone, it is calculated that nearly half of the O_2^- generated was utilized for the formation of cysteinyldopas. The remaining half may be decomposed through the spontaneous dismutation which proceeds rapidly at pH 7.4 [14].

Superoxide dismutase at 50 μg/ml completely inhibited the conjugation of dopa with cysteine (Table 2). The hypoxanthine–xanthine oxidase system produces not only O_2^- but also hydroxyl radical (·OH) through the Haber-Weiss reaction when trace amounts of iron complexes are present [15, 16]. As shown in Table 2, both mannitol and formate, scavengers of ·OH [15], had no effect at a concentration 100 times that of dopa. These results indicate that the active oxygen responsible for the present reaction was in fact O_2^- but not ·OH.

Inhibition of O_2^--dependent reduction of nitro-blue tetrazolium by dopa and 5-S-cysteinyldopa. Figure

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Table 1. Rates of $O_2^{\cdot-}$-mediated formation of cysteine conjugates from dopa and 5-*S*-cysteinyldopa at pH 7.4*

Compounds (μM)	Products (μM/5 min)
Dopa (500)	5-*S*-Cysteinyldopa (19.1 ± 0.4)
+ Cysteine (1000)	Cystine (1.6 ± 0.2)
5-*S*-Cysteinyldopa (500)	2,5-*S,S*-Dicysteinyldopa (16.8 ± 0.4)
+ Cysteine (1000)	Cystine (7.0 ± 0.6)

* The reaction mixture contained L-dopa or 5-*S*-cysteinyldopa, L-cysteine, 100 μM L-glutamic acid (internal standard for amino acid analysis), 200 μM hypoxanthine, and catalase (20 μg/ml) in 4 ml of 0.05 M potassium phosphate buffer (pH 7.4). The reaction was started by adding 76 μg/ml xanthine oxidase and continued at 30° for 5 min. The reaction was stopped by adding 1 ml of 20% trichloroacetic acid containing 5 mM EDTA, and the mixture was assayed for amino acid with a JEOL JLC-6AH amino acid analyzer. Blank experiments were carried out in the absence of xanthine oxidase. Values are expressed as mean ± S.D. for two separate experiments; each reaction mixture was assayed twice.

1 shows the effects of dopa and 5-*S*-cysteinyldopa on the production of formazan from nitro-blue tetrazolium by $O_2^{\cdot-}$ at pH 7.4. Addition of EDTA had little effect, as in the case of a similar experiment at pH 7.8 [17]. Cysteine produced a weak accelerating effect, perhaps due to the removal of traces of Cu^{2+} and other metals present in the reaction mixture. Dopa and 5-*S*-cysteinyldopa significantly inhibited the formazan production.

The results shown in Fig. 1 indicate that 5-*S*-cysteinyldopa reacted with $O_2^{\cdot-}$ at a faster rate than dopa. This seemed to contradict the results that 5-*S*-cysteinyldopa gave a lower yield of conjugation product than dopa (Table 1). This apparent discrepancy may be explained by assuming that 5-*S*-cysteinyldopa has a higher reactivity with $O_2^{\cdot-}$ than dopa, but 5-*S*-cysteinyldopaquinone has a lower reactivity with cysteine than dopaquinone.

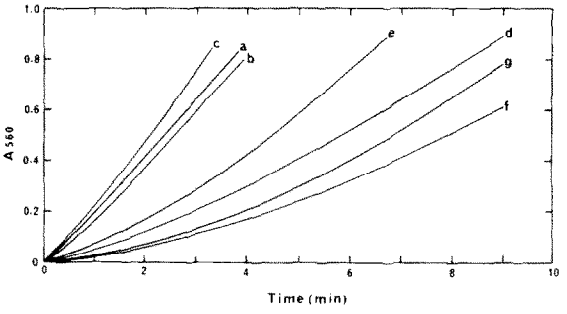

Fig. 1. Effects of dopa, 5-*S*-cysteinyldopa and cysteine on reduction of nitro-blue tetrazolium by $O_2^{\cdot-}$ at pH 7.4. A mixture of 200 μM hypoxanthine, 500 μM nitro-blue tetrazolium, 20 μg/ml catalase, and EDTA or the amino acid(s) described below in 0.05 M potassium phosphate buffer (pH 7.4) was incubated at 30° in the presence of 76 μg/ml xanthine oxidase. (a) No addition. (b) Plus 1000 μM EDTA. (c) Plus 1000 μM L-cysteine. (d) Plus 500 μM L-dopa. (e) Plus 500 μM L-dopa and 1000 μM L-cysteine. (f) Plus 500 μM 5-*S*-cysteinyldopa. (g) Plus 500 μM 5-*S*-cysteinyldopa and 1000 μM L-cysteine. Each reaction was carried out twice, giving almost identical results.

DISCUSSION

Superoxide radical ($O_2^{\cdot-}$) is generated in many biological processes [16]. The present study shows that $O_2^{\cdot-}$ can mediate the conjugation of dopa and 5-*S*-cysteinyldopa with cysteine at a physiological pH. The rates of oxidation of the catechols by $O_2^{\cdot-}$ appear to be comparable to that of reduction of nitro-blue tetrazolium by $O_2^{\cdot-}$. On the other hand, cysteine cannot react with $O_2^{\cdot-}$ at an appreciable rate. This is in agreement with a recent report that cysteine has a relatively small rate constant for the reaction with $O_2^{\cdot-}$ [18].

There have been a number of reports suggesting that $O_2^{\cdot-}$ can mediate covalent binding of catechols

Table 2. Effects of superoxide dismutase and hydroxyl radical scavengers on $O_2^{\cdot-}$-mediated formation of 5-*S*-cysteinyldopa*

Reagent added	Concentration	5-*S*-Cysteinyldopa (μM/5 min)	% Inhibition
None	–	25.6 ± 0.1	0
Superoxide dismutase	10 μg/ml	3.3 ± 0.0	87
	50 μg/ml	0.2 ± 0.0	99
Mannitol	50 mM	26.0 ± 0.4	0
Sodium formate	50 mM	25.2 ± 0.3	2

* The reaction mixture was as described in Table 1, except that the concentration of xanthine oxidase was 100 μg/ml. 5-*S*-Cysteinyldopa was quantified as described in Table 1. Values are expressed as mean ± S.D. for two separate experiments; each reaction mixture was assayed twice.

with proteins through sulfhydryl groups [2]. However, direct evidence has been lacking. Our results suggest that such binding reaction is chemically feasible.

Normal cells have defence mechanisms against cytotoxic $O_2^{\cdot-}$ and the quinone oxidation products; the former is scavenged rapidly by superoxide dismutase or ascorbic acid [19], and the latter by glutathione or ascorbic acid [5]. Therefore, it seems likely that catechols exert greater cytotoxicity in cells where such defence is not sufficient.

Finally, the present results suggest that the higher antitumour activity of 5-*S*-cysteinyldopa than dopa [8] should be attributed to mechanisms other than the $O_2^{\cdot-}$-mediated conjugation with sulfhydryl groups of proteins.

REFERENCES

1. M. M. Wick, *Cancer Treat. Rep.* **65**, 861 (1981).
2. E. Dybing, S. D. Nelson, J. R. Mitchell, H. A. Sasame and J. R. Gillette, *Molec. Pharmac.* **12**, 911 (1976).
3. A. Rotman, J. W. Daly and C. R. Creveling, *Molec. Pharmac.* **12**, 887 (1976).
4. D. G. Graham, S. M. Tiffany, W. R. Bell Jr. and W. F. Gutknecht, *Molec. Pharmac.* **14**, 644 (1978).
5. D. C. S. Tse, R. L. McCreery and R. N. Adams, *J. med. Chem.* **19**, 37 (1976).
6. G. Prota, *J. invest. Dermat.* **75**, 122 (1980).
7. G. Agrup, P. Agrup, T. Andersson, L. Hafstrom, C. Hansson, S. Jacobsson, P.-E. Jonsson, H. Rorsman, A.-M. Rosengren and E. Rosengren, *Acta Dermatovener. (Stockholm)* **59**, 381 (1979).
8. K. Fujita, S. Ito, S. Inoue, Y. Yamamoto, J. Takeuchi, M. Shamoto and T. Nagatsu, *Cancer Res.* **40**, 2543 (1980).
9. S. Ito and G. Prota, *Experientia* **33**, 1118 (1977).
10. S. Ito and K. Fujita, *Biochim. biophys. Acta* **672**, 151 (1981).
11. S. Ito, S. Inoue, Y. Yamamoto and K. Fujita, *J. med. Chem.* **24**, 673 (1981).
12. J. M. McCord and I. Fridovich, *J. biol. Chem.* **244**, 6049 (1969).
13. C. Beauchamp and I. Fridovich, *Analyt. Biochem.* **44**, 276 (1971).
14. Y. Sawada and Y. Yamazaki, *Biochim. biophys. Acta* **327**, 257 (1973).
15. B. Halliwell, *FEBS Lett.* **92**, 321 (1978).
16. W. Bors, M. Saran and G. Czapski, in *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase* (Eds W. H. Bannister and J. V. Bannister), p. 1. Elsevier, Amsterdam (1980).
17. B. Halliwell, *FEBS Lett.* **56**, 34 (1975).
18. B. H. J. Bielski and G. G. Shiue, in *Oxygen Free Radicals and Tissue Damage* (Ciba Foundation Symposium 65), p. 43. Excerpta Medica, Amsterdam (1979).
19. M. Nishikimi, *Biochem. biophys. Res. Commun.* **63**, 463 (1975).