IRREVERSIBLE BINDING OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYTRYPTOPHAN METABOLITES TO RAT LIVER MICROSOMAL PROTEIN

Tomihiko UEMURA, Hiroshi MATSUSHITA, Masako OZAWA, Andrea FIORI[+] and Enzo CHIESARA

Division of Neurochemistry, Psychiatric Research Institute of Tokyo 2-1-8, Kamikitazawa, Setagaya-ku, Tokyo, 156, Japan and *[+]Institute of Pharmacology, University of Milan, Via Vanvitelli 32, Milan, Italy*

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1. Introduction

The main metabolic fate of oxidative deamination of the indole alkylamines by MAO has been well established [1]. The oxidation of the phenolic hydroxyl groups of indole nucleus is, however, poorly elucidated, although the enzymatic oxidation of 5-HT by ceruloplasmin [2,3], by invertebrate hydroxyindole oxidase [4,5] and by mammalian cytochrome oxidase [6,7], and its non-enzymatic oxidation by denatured oxyhaemoglobin [8], by ferricytochrome *c* [9] and by inorganic oxidants such as copper and $AgNO_3$ [10] have been reported.

Metabolites of catecholamines have been shown [11,12] to bind irreversibly to microsomal membrane protein in the presence of NADPH and O_2. Adrenochrome is reported [13] to be a potent inhibitor of microsomal hydroxylation reaction. Since adrenochrome has indole nucleus in its structure, we examined the in vitro effect of other indole compounds on the hydroxylation reaction. During the course of this study we noticed that indole compounds having a hydroxyl group at position 5 consume both NADPH and molecular oxygen in the presence of microsomes.

This paper describes that rat liver microsomes catalyze NADPH-dependent, irreversible binding of metabolites of 5-HT and 5-HTP to microsomal protein and that the binding is inhibited clearly by superoxide dismutase and slightly by both CO and phenylisocyanide, suggesting that cytochrome *P*-450 is not required absolutely for the binding of 5-hydroxyindoles to microsomal protein.

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; 5-HI, 5-hydroxyindole; 5-HIA, 5-hydroxyindoleacetic acid; 6-HT, 6-hydroxytryptamine; MAO, monoamine oxidase; SOD, superoxide dismutase

2. Materials and methods

Microsomes were prepared by the method in [14] from the livers of male Wistar rats (200–300 g) fasted for 12 h. The livers were perfused with an ice-cold 1.15% KCl solution to remove as much haemoglobin as possible. The reaction mixture (final vol. 1 ml) for the binding studies contained 0.1 M Tris–HCl buffer (pH 7.8), 1 mg microsomal protein, 0.4 mM 5-HT or 5-HTP, 5-H[^{14}C]T or 5-H[^{14}C]TP (each 1 μCi), 1.5 mM KCN (to inhibit contaminating cytochrome oxidase) and NADPH generating system composed of 0.6 mM NADP, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 7 U glucose-6-phosphate dehydrogenase, and was incubated at 35°C for 30 min with constant shaking in air. The reaction was stopped by adding 0.5 ml 0.5 M borate buffer (pH 10.1) then 6 ml ethyl acetate. The precipitate was collected by washing with 6 ml absolute ethanol (once), 3 ml each of 90% methanol (3 times), 80% methanol (3 times) and 70% ethanol (once) by repeating suspension and centrifugation. No radioactivity was detected in the last ethanol wash. The thoroughly washed precipitate was dissolved in 1 ml 1 N NaOH, 0.5 ml aliquot was mixed with 20 ml Bray's scintillation solution [15] and the radioactivity was measured. Protein was measured according to [16].

Oxygen consumption was measured at 35°C with

a Clark electrode in an oxygen monitor Mod. 53 YSI. NAD(P)H oxidation was assayed at 35°C by measuring the ΔA_{340} in a Shimadzu UV-300 spectrophotometer.

Superoxide dismutase was purified as in [17]. Phenylisocyanide was synthesized by Dr Y. Ichikawa, Dept Biochemistry, Osaka University Medical School, and kindly supplied for the present experiments. 6-HT was purchased from Sigma Chemical Co.

3. Results

3.1. *Effect of indole compounds on the NADPH oxidase activity and O_2 consumption*

Microsomal oxidation of NADPH and O_2 consumption were greatly stimulated by adding 5-HT as a representative substrate both in the presence (system A) and absence (system B) of KCN (fig.1). Cyanide increased the apparent amount of O_2 consumed by ~2-fold. Under anaerobic conditions, NADPH was not oxidized even in the presence of 5-HT. NADH was also active as electron donor, although the initial lag period was much longer than that of NADPH. Concerning the substrate specificity, all 5-hydroxyindole compounds were active (table 1). Of considerable interest was the observation that 6-HT was also active as 5-HT. Neither simple indole compounds having no hydroxyl group at position 5 (tryptophan, tryptamine, indole) nor those of which hydroxyl group is blocked by methylation (melatonin) could stimulate the oxidation of NADPH and the consumption of O_2 under our experimental conditions, although the existence in liver microsomes of the tryptamine [18] and melatonin [19] hydroxylating enzyme system(s) that requires NADPH and O_2 has been reported.

Table 1
Dependence of the stimulation of NADPH oxidation and O_2 consumption by rat liver microsomes on various indole compounds

	NADPH oxidation	O_2 consumption
	(nmol NADPH oxidized/ 8 min/mg protein)	(nmol O_2/8 min/ mg protein)
None	36.2	56.9
5-HT	242.0	277.5
N-Acetyl-5-HT	213.6	316.1
Bufotenine	217.0	310.7
5-HTP	215.7	289.3
5-HI	235.6	291.4
5-HIA	179.1	267.8
6-HT	278.3	321.1
Tryptophan	36.2	53.6
Tryptamine	27.6	41.8
Melatonin	35.8	48.2
Indole	38.2	42.9

NADPH oxidation and O_2 consumption were measured in a reaction mixture containing 1 mg microsomal protein, 0.1 M Tris–HCl buffer (pH 7.8), 1.5 mM KCN, 0.4 mM indole compounds and 150 μM NADPH in 3.0 ml final vol., at 35°C. The values were given as extent calculated from such curves as in fig.1

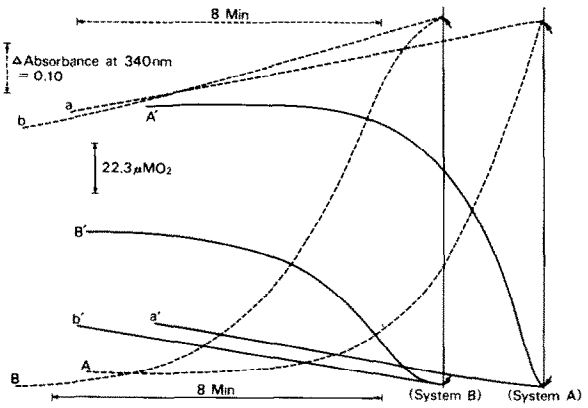

Fig.1. Effect of 5-HT on NADPH oxidation and O_2 consumption in the presence or absence of KCN by rat liver microsomes under aerobic conditions. For the measurement of both NADPH oxidation and O_2 consumption, the reaction mixture, in 3 ml final vol. contained 1 mg microsomal protein, 0.1 M Tris–HCl buffer (pH 7.8), 0.4 mM 5-HT and 150 μM NADPH added at the arrows in the presence (system A) or absence (system B) of 1.5 mM KCN at 35°C. (· · ·) NADPH oxidation; (——) O_2 uptake, A, A', B, B'; in the presence of 5-HT, a, a', b, b'; in the absence of 5-HT.

3.2. *Involvement of superoxide anion radical ($O_2^{\cdot-}$) in 5-HT-dependent NADPH oxidation and O_2 consumption*

As shown in fig.2, the pre-existence of SOD showed the almost complete inhibition of both 5-HT-stimulated O_2 uptake and NADPH oxidation (curve a, A). The addition of SOD during the steady state, however, inhibited only the 5-HT-stimulated O_2 uptake and had no effect on 5-HT-dependent NADPH oxidation (curve b, B). These results suggest the total and partial involvements of $O_2^{\cdot-}$ in the 5-HT-dependent O_2 consumption and NADPH oxidation, respectively.

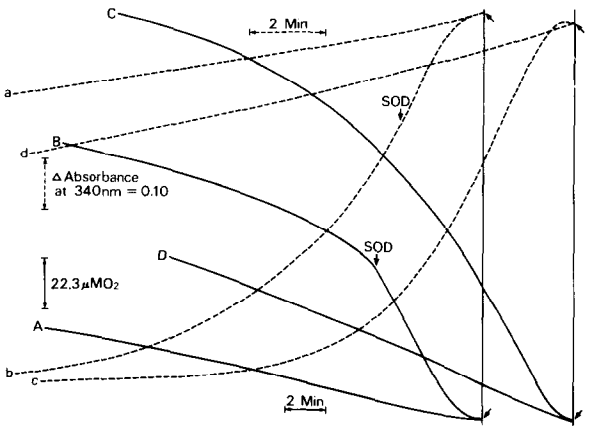

Fig.2. Effect of superoxide dismutase on microsomal 5-HT-dependent NADPH oxidation and O_2 consumption. The experimental conditions were the same as for fig.1, except that NADPH was added at 0.6 mM final conc. for O_2 consumption at the arrows. The amount of SOD added was 80 μg. KCN was not added in all reaction mixtures. (· · ·) NADPH oxidation; (——) O_2 uptake; (a, A) pre-existence of SOD; (b, B) addition of SOD where indicated; (c, C) in the absence of SOD; (d, D) in the absence of 5-HT.

3.3. *The formation of protein-bound radioactive metabolite(s) and the stoichiometry of NADPH oxidation, O_2 consumption and the protein-bound product formation*

The protein-bound radioactivity, which was not eliminated by extraction with organic solvent, was detected by incubating 5-HT or 5-HTP with microsomes in the presence of a NADPH generating system and O_2 (table 2). Without NADPH, the binding was slight. The quantity of metabolite(s) of 5-hydroxyindoles bound to 1 mg microsomal protein was nearly within the same order as that of α-methyldopa [12]. The binding was inhibited slightly by both CO and phenylisocyanide. This means that cytochrome P450 appears not to be a main component in the binding reaction. The binding was, however, inhibited clearly by $O_2^{\cdot-}$ scavengers, GSH and ascorbate as in the case of similar binding studies [11,12,20].

The stoichiometry of the reaction was calculated from fig.1 and table 2. The amounts of NADPH and O_2 consumed in the reaction mixture for 8 min was ~258 nmol and 135 nmol, respectively, in the absence of KCN, giving a NADPH/O_2 ratio of about 2/1 which approached 1/1 by the inclusion of KCN in the reaction mixture. It was difficult to estimate exactly the ratio of O_2 consumption to the protein-bound product formation because of the different experimental conditions (see section 2). In the presence of KCN, however, the initial rates were ~100–130 and 0.9–1.0 nmol/min/mg protein for O_2 consumption and the product formation, respectively. Thus the ratio was ~1:0.01.

4. Discussion

Different from the hydroxylation reaction, the present study indicates that oxygen atom is not inserted

Table 2
Irreversible binding of 5-H[^{14}C]T and 5-H[^{14}C]TP metabolites to rat liver microsomal protein and the effects of various binding conditions

		5-HT		5-HTP	
		(nmol/mg protein/30 min)	(%)	(nmol/mg protein/30 min)	(%)
Complete system		28.81 ± 2.52 (8)	100	19.94 ± 3.73 (8)	100
−NADPH generating system		3.18 ± 0.34 (3)	11	3.40 ± 0.42 (3)	17
CO/O_2	(80/20)	21.89 ± 3.82 (3)	76	15.41 ± 0.45 (3)	77
Phenylisocyanide	1.0 mM	23.04 ± 1.88 (3)	80	17.24 ± 3.49 (4)	86
Tiron	1.0 mM	13.40 ± 3.62 (4)	47	6.28 ± 1.72 (3)	31
SOD	75 μg/ml	1.45 ± 0.03 (3)	5	1.11 ± 0.06 (3)	6
GSH	1.0 mM	3.17 ± 1.25 (3)	11	2.41 ± 0.87 (3)	12
Ascorbic acid	1.0 mM	1.18 ± 0.22 (3)	4	0.81 ± 0.22 (3)	4

The experimental conditions are in section 2. Values are expressed as mean ± SD from separate experiments. When the number of determinations (in brackets) was < 5, SD = $[\Sigma(\text{dev.})^2/n-1]^{1/2}$

into the substrate molecule, but $O_2^{\cdot-}$ derived from O_2 serves solely as oxidizer so as to be transformed into H_2O_2 (data not shown). H_2O_2 is then metabolized by catalase contaminated in microsomal fraction, thus giving the ratio of 2:1 for NADPH oxidation to O_2 consumption. In the presence of KCN, however, this ratio approaches 1:1 because of the inhibition of catalase action by cyanide. The catalysis of the oxidation of 5-hydroxyindoles may involve cycling between fully reduced (quinoneimine) and half-reduced (semiquinone) forms, thus consuming a large amount of O_2. Only a small portion of either form may bind to microsomal protein. This may account for the ratio of 1:0.01 for O_2 consumption to the formation of protein-bound product. The inhibitory effect of SOD on 5-HT-stimulated O_2 uptake (fig.2), even when added during the steady state, suggests that a certain intermediate once formed (probably semiquinone) may also react with $O_2^{\cdot-}$.

The physiological meaning of this binding reaction is the subject of ongoing studies. A recent report of the formation of peptido–5-HT complex by hypothalamic tissue extracts [21] is of interest in relation to the present binding studies of 5-hydroxyindoles. In fact, our preliminary work indicates that NADPH- and O_2-dependent, irreversible binding of 5-hydroxyindoles takes place in the brain microsomal preparation as in the case of catecholamines [20].

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