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Note

Identification of 5-S- and 2-S-cysteinyldopamine and 5-S-glutathionyldopamine formed from dopamine by high-performance liquid chromatography with electrochemical detection

SHOSUKE ITO* and KEISUKE FUJITA

Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen Health University, Toyoake, Aichi 470-11 (Japan)

MASANORI YOSHIOKA

Setsunan University, Faculty of Pharmaceutical Sciences, Osaka 573-01 (Japan)

and

DOROTA SIENKO* and TOSHIHARU NAGATSU**

Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227 (Japan)

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Boonyarat et al [1] previously reported a sensitive assay for the non-specific N-methyltransferase activity [2] in rat tissues by high-performance liquid chromatography with electrochemical detection (HPLC—ED). The principle of the assay was based on the HPLC—ED of N-methyldopamine formed from dopamine Non-specific N-methyltransferase catalyses the formation of N-methylamines from various aromatic amines and is distributed in many tissues including the lung [2]. One of the present authors (D S) found that although

*Present address Department of Chemistry, Marie-Curie Skolodowska University, Lublyn, Poland
**Present address Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466, Japan

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the product had an identical retention time to that of N-methyldopamine under previously reported HPLC conditions [1], it was different from and separated from N-methyldopamine, and that another unknown product was also detected by HPLC—ED.

Later experiments proved that the product was formed even in the absence of exogenous S-adenosylmethionine, and that the reaction was dependent on the presence of oxygen.

In the present paper, we report that the product previously identified as N-methyldopamine is 5-S-cysteinyldopamine and that 2-S-cysteinyldopamine and 5-S-glutathionyldopamine are also formed from dopamine in the reaction mixture containing dopamine and rat tissues.

EXPERIMENTAL

Chemicals

Pargyline, dopamine, N-methyldopamine, 3,4-dihydroxybenzylamine (DHBA), L-cysteine and mushroom tyrosinase (EC 1.14 18.1, 2000 U/mg) were purchased from Sigma (St Louis, MO, U S.A) All other chemicals were of analytical grade and obtained from Wako (Osaka, Japan)

Preparation of cysteinyldopamines

The method of Ito and Prota [3] for the preparation of cysteinyldopas was adapted with minor modifications. A solution of 0 5 mmol of dopamine · HCl and 1 mmol of L-cysteine in 50 ml of water was adjusted to pH 6.8 by adding crystals of disodium hydrogen phosphate After the addition of 25 mg of mushroom tyrosinase, the mixture was stirred at room temperature (24° C) The course of the oxidation was monitored every 30 min by HPLC under the conditions described below After 3 h, when dopamine was completely consumed, the oxidation was stopped by adding 5 ml of 6 *M* hydrochloric acid The reaction mixture was passed through a column (8 × 2 0 cm I D.) of Dowex 50W-X2-H^+ (200—400 mesh, equilibrated with water) After washing with 100 ml of 0 5 *M* hydrochloric acid, the products were eluted with 3 *M* hydrochloric acid, and 50-ml fractions were collected and monitored by HPLC Fractions 1—7, which contained the cysteinyldopamines, were combined and evaporated to dryness in a rotary evaporator. The residue, taken up in 2 *M* hydrochloric acid, was rechromatographed on a column (23 × 2 0 cm I.D.) of Dowex 50W-X2-H^+ (200—400 mesh, equilibrated with 2 *M* hydrochloric acid) and 20-ml fractions were collected and monitored by UV spectrophotometry Fractions 44—52, 53—64, 65—94 and 112—144 contained 6-S-, 2-S-, 5-S-cysteinyldopamine and 2,5-S,S′-dicysteinyldopamine, respectively Assuming that they have molar extinction coefficients identical to those of the corresponding cysteinyldopas [4], their yields were 1.2, 7 9, 79 and 3 8%, respectively Evaporation of fractions 54—63 and 66—92 gave 2-S- and 5-S-cysteinyldopamine · 2HCl as a pale-green powder They were further purified by passing separately through a column (5 × 2.0 cm I.D) of Dowex 50W-X2-H^+ (200—400 mesh, equilibrated with 3 *M* hydrochloric acid) and eluting with 3 *M* hydrochloric acid (20 ml per fraction) Evaporation of fractions containing the desired product (frations 6– 9 for the 2-S isomer and fractions 7– 12

for the 5-S isomer) gave 16 3 mg (7.6% yield) of 2-S-cysteinyldopamine · 2HCl and 137 mg (72% yield) of 5-S-cysteinyldopamine · 2HCl as a colourless powder. They were practically pure, as judged by HPLC and UV spectrophotometry.

Preparation of glutathionyldopamines

A solution of 0 05 mmol of dopamine · HCl and 0 1 mmol of glutathione in 5 ml of water was adjusted to pH 6.8 by adding crystals of disodium hydrogen phosphate. After the addition of 2 5 mg of mushroom tyrosinase, the mixture was stirred at room temperature After 2 h, the oxidation was stopped by adding 0 5 ml of 6 *M* hydrochloric acid. The major product appearing at 4 2 min under the following HPLC conditions was assumed to be 5-S-glutathionyldopamine, in analogy with the tyrosinase oxidation of dopamine with glutathione [5]

Chromatography

A Yanaco Model L-5000 liquid chromatograph (Yanagimoto, Kyoto, Japan) was used with a Yanaco Model VMD-101A electrochemical detector. The detector was set at +600 mV vs an Ag/AgCl reference electrode and sensitivity was 4 nA f s Separation was achieved on a C_{18} reversed-phase column (Yanaco ODS-T, particle size 10 μm, 250 × 4 mm I D) at 50°C The mobile phase was acetonitrile—0.1 *M* sodium citrate buffer, pH 5.0, containing 6 mmol/l sodium octanesulphonate and 1 mmol/l Na_2EDTA (60:940). The flow-rate was 1.0 ml/min. Under these chromatographic conditions, retention times were 5-S-glutathionyldopamine, 4 2 min, 2-S-cysteinyldopamine, 6.5 min, DHBA, 8 6 min, 5-S-cysteinyldopamine, 10 9 min; dopamine, 12.7 min, N-methyldopamine, 15 8 min

Sample preparation

Rates were decapitated, and tissues were removed immediately and homogenized in 5 vols of 0 25 *M* sucrose in a glass homogenizer

Procedure

A typical incubation mixture for dopamine consisted of the following components in a total volume of 250 μl (final concentrations in parentheses) in a microcentrifuge tube (1 5 ml volume) 10 μl of 0 01 *M* pargyline (0 4 m*M*), 50 μl of 0.5 *M* Tris–hydrochloric acid buffer (pH 7 25, 0 1 *M*), 10 μl of 0 45 m*M* dopamine (18 μ*M*), 50 μ*l* of tissue homogenate (8 mg tissue) and water Although S-adenosylmethionine stimulated the reaction, it was omitted from the incubation mixture, since enough products from dopamine were produced in the absence of S-adensylmethionine The boiled homogenate was a lung homogenate heated in a boiling-water bath for 5 min In the experiments designated as O_2 exclusion, air was replaced with argon. In the experiments designated as + cysteine, 10 μl of 0 9 m*M* L-cysteine (36 μ*M*) were added.

After incubation for 60 min at 37°C, the reaction was stopped by adding 100 μl of 1.4 *M* perchloric acid, and to this were added 10 μl of 2 μ*M* DHBA (20 pmol) as an internal standard The mixture was left for 10 min in an ice-bath and centrifuged (10 000 *g* for 2 min) A 300-μl aliquot of the supernatant

was transferred to a microcentrifuge tube (1.5 ml volume) containing 50 mg of acid-washed alumina and 100 μl of 2% (w/v) sodium metabisulphite. Catechols were then adsorbed onto alumina by adding 1.0 ml of 1.5 *M* Tris—hydrochloric acid buffer containing 2% (w/v) Na_2EDTA (pH 8 8) and by immediately shaking for 5 min on a JASCO MT-30 multi-tube mixer (Tokyo, Japan). After centrifugation, alumina was washed twice with ca. 1.5 ml of water. Catechols were then eluted with 200 μl of 0.4 *M* perchloric acid by shaking for 2 min After centrifugation, 100 μl of the supernatant were injected into the HPLC system. For the standard, the tissue homogenate was replaced with water, and 100 μl of 1 4 *M* perchloric acid, 10 μl of each 2 μM 5-S- and 2-S-cysteinyldopamine (20 pmol each) and 10 μl of 2 μM DHBA (20 pmol) were immediately added without incubation

RESULTS

Fig 1A shows a typical chromatogram of the dopamine incubation with rat lung homogenate.When compared with a chromatogram of the mixture without incubation (Fig. 1B), two unknown peaks, UN1 and UN2, were observed. These compounds were assumed to be 2-S- and 5-S-cysteinyldopamine on the following basis they were shown to be oxidation products of dopamine, and their relative ratios of production and relative position in the chromatogram resembled those of 2-S- and 5-S-cysteinyldopa [3, 6] This assumption was confirmed by direct comparison of their retention times with those of

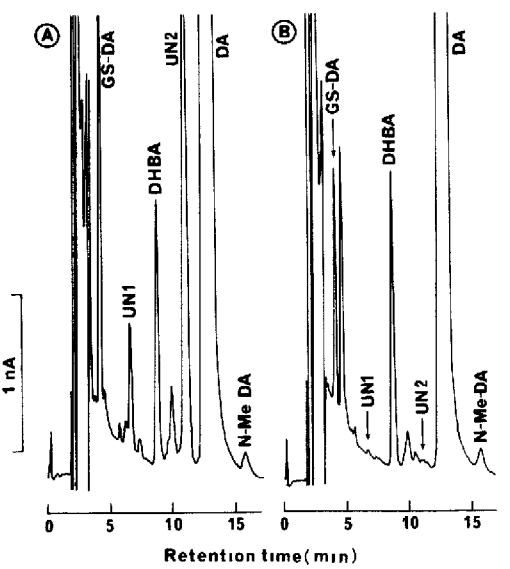

Fig 1 Typical chromatogram of an incubation mixture of dopamine with the homogenate of rat lung The incubation mixture consisted of 0 4 m*M* pargyline, 18 μM dopamine, 0 1 *M* Tris—hydrochloric acid buffer (pH 7 25) and 50 μl of lung homogenate (8 mg tissue) in a 250-μl volume (A) Experimental incubation with homogenate for 1 h at 37°C (B) No incubation, otherwise, identical to A DHBA (20 pmol) was added as an internal standard Peaks GS-DA = 5-S-glutathionyldopamine, UN1 = 2-S-cysteinyldopamine, UN2 = 5-S-cysteinyldopamine, DA = dopamine, N-Me-DA = N-methyldopamine

2-S- and 5-S-cysteinyldopamine, which were prepared by tyrosinase oxidation of dopamine in the presence of cysteine (see Experimental) Their identification was further supported by HPLC comparison under the conditions used for the separation of cysteinyldopa isomers [6]. The molar ratio of 5-S to 2-S isomer was 7 1 1 in the incubation mixture with lung homogenate.

As shown in Fig. 1A and B, N-methyldopamine was detected both in experiment and control, and did not increase by incubation with lung homogenate Addition of S-adenosylmethionine to the reaction mixture did not increase the peak height of N-methyldopamine.

The conditions by which cysteinyldopamines were formed were then studied and the results are summarized in Table I Surprisingly, the boiled homogenate retained about 60% of the original activity (Exp. 3). Oxygen was required for a higher activity (Exp. 4).

The formation of cysteinyldopamines was also observed when dopamine was incubated with cysteine in the absence of the homogenate (Exp 5). The molar ratio of 5-S to 2-S isomer was 8 2 1 in this mixture. The non-enzymic production of cysteinyldopamines was mostly dependent on oxygen, as indicated in Exps. 6 and 7 Addition of the homogenate to the incubation mixture decreased the 5-S-cysteinyldopamine production (Exp 8). On the other hand, addition of the boiled homogenate stimulated the reaction by ca. 40% (Exp. 10) These results may be ascribed to the presence of inhibitor(s) in the fresh homogenate but not in the boiled homogenate.

Glutathione (reduced) is the most abundant non-protein thiol compound in tissues [7]. Thus, the effect of glutathione on the production of 5-S-cysteinyldopamine by non-enzymic oxidation was studied As shown in Table II, a ten-fold excess of glutathione inhibited the rate of oxidation to one sixth of the original value (Exp 3) The rate of 5-S-cysteinyldopamine production was within the same range as the original one when ten-fold excess of L-cysteine

TABLE I

5-S-CYSTEINYLDOPAMINE PRODUCTION UNDER VARIOUS CONDITIONS

The incubation mixture contained 0 4 m*M* pargyline, 18 μM dopamine, 0 1 *M* Tris—hydrochloric acid buffer (pH 7 25) and 50 μl of lung homogenate (8 mg tissue) in a 250-μl volume. In Exps 5—10, 36 μM L-cysteine was included For details, see Experimental The results are expressed in pmol/h 5-S-cysteinyldopamine formed in the mixture and represent means ± S D for two duplicate analyses

Experiment No	Conditions	5-S-Cysteinyldopamine production (pmol/h)
1	+ Homogenate	33 ± 2 5
2	+ Homogenate, no incubation	1 6 ± 0 7
3	+ Boiled homogenate	22 ± 2 4
4	+ Homogenate, O_2 exclusion	18 ± 3 0
5	— Homogenate, + cysteine	198 ± 35
6	— Homogenate, + cysteine, no incubation	25 ± 16
7	— Homogenate, + cysteine, O_2 exclusion	30 ± 8 6
8	+ Homogenate, + cysteine	72 ± 18
9	+ Homogenate, + cysteine, no incubation	31 ± 9 2
10	+ Boiled homogenate, + cysteine	285 ± 66

TABLE II

EFFECT OF GLUTATHIONE ON 5-S-CYSTEINYLDOPAMINE PRODUCTION

The incubation mixture contained 0 4 m*M* pargyline, 18 μM dopamine, the thiol compound(s) indicated above and 0 1 *M* Tris—hydrochloric acid buffer (pH 7 25) in a 250-μl volume Exp 1 in this table is identical to Exp 5 in Table I For details, see Experimental The results are expressed in pmol/h 5-S-cysteinyldopamine formed in the mixture and represent means ± S D for two duplicate analyses

Experiment No	Conditions	5-S-Cysteinyldopamine production (pmol/h)
1	36 μM L-Cysteine	189 ± 91
2	360 μM Glutathione	0 0
3	360 μM Glutathione + 36 μM L-cysteine	30 ± 8 0
4	360 μM Cysteine	161 ± 49

was added (Exp 4). These results indicate that inhibition by excess glutathione was due to the competition between the two thiol compounds in the addition reaction to a reactive intermediate such as dopamine—quinone [8]. In fact, a peak corresponding to 5-S-glutathionyldopamine was detected in the chromatograms of the incubation mixtures containing glutathione (Exps. 2 and 3) The same compound was produced on incubation of dopamine with lung homogenate (Fig. 1A)

Various tissues from a rat were analysed for 5-S-cysteinyldopamine production The brain, heart, spleen and kidney had activities of 57, 5 4, 96 and 36 pmol/h per 8 mg of tissue, respectively. 5-S-Glutathionyldopamine was also detected in the incubation mixtures of these tissues, except for the kidney

DISCUSSION

The present study shows that the two unknown compounds, UN1 and UN2, produced on incubation of dopamine with rat tissues are 2-S- and 5-S-cysteinyldopamine, respectively. Enzyme(s) may not play a major role in the oxidation We have previously shown that conjugation of dopa with cysteine can be mediated by a variety of biological oxidation systems such as tyrosinase-O_2 [3], peroxidase-H_2O_2 [9], superoxide radical [10], hydroxyl radical [11] and iron chelate [12]. Agrup et al [13] have shown that methaemoglobin also catalyses the conjugation of dopa with cysteine Although tissue components responsible for the oxidation were not identified, it is apparent that exogenous dopamine can bind easily with endogenous cysteine by non-enzymic oxidation, perhaps catalysed by endogenous iron ions

There have been few reports on the tissue contents of cysteine. According to Gaitonde [14], rat blood, brain and liver contain cysteine at a level of 31—95 nmol/g of tissue. This concentration of endogenous cysteine appears to be enough for the observed cysteinyldopamine formation. It is known that glutathione reacts with dopamine—quinone two thirds as fast as cysteine [8] Since glutathione is the chief thiol compound in tissues, glutathione conjugates of dopamine should be the major products of incubation of dopamine with tissue homogenates Agrup et al [15] have shown that glutathione conjugate(s)

of dopa can be split to cysteinyldopa(s) on incubation with guinea pig kidney homogenate but not with lung homogenate Thus, in tissue containing high levels of γ-glutamyltranspeptidase and peptidases, cysteinyldopamines may be formed also via glutathionyldopamines

Ascorbic acid can reduce dopamine—quinone back to dopamine at a rate comparable to that of the reaction with glutathione [8] Thus, the fact that 5-S-cysteinyldopamine production in the presence of cysteine increased four-fold after boiling the homogenate may be ascribed to the decrease of ascorbic acid as an inhibitor of the reaction

From the results presented in this paper, it appears that the rate of cysteinyl-dopamine production on incubation of dopamine may be affected by levels of many tissue components, such as cysteine, glutathione, ascorbic acid, iron ions, γ-glutamyltranspeptidase and other enzymes.

Finally, the present study indicates the possibility that cysteinyldopamines are formed in tissues where dopamine is present at a high level

Since N-methyldopamine formation in the reaction mixture with lung homogenate, even in the presence of exogenous S-adenosylmethionine, was not significant, the previously reported method for non-specific N-methyl-transferase [1] may not be applicable, at least to crude enzyme preparations.

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