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Neurotoxicity of free-radical-mediated serotonin neurotoxin in cultured embryonic chick brain neurons

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Abstract

Exposure of serotonin (5-HT) to oxygen-derived free-radical-generating system, xanthine oxidase-hypoxanthine or to a Fenton reaction results in the formation of the neurotoxin, tryptamine-4,5-dione. In cultured embryonic chick brain neurons, incubation of tryptamine-4,5-dione or its ethyl carbonate derivative resulted in a dose-dependent neurotoxicity (1–100 μM). The addition of sulfhydryl compound, glutathione at 2 or 10 μM significantly enhanced the toxicity induced by 10 μM tryptamine-4,5-dione. On the contrary, glutathione at 10 μM decreased the neurotoxic effect caused by 10 μM 5,6- and 5,7-dihydroxytryptamine in the cultured neurons. The toxicity resulted from 5,6- and 5,7-dihydroxytryptamine could be fully prevented by a 5-HT uptake inhibitor, fluoxetine. However, the toxicity caused by tryptamine-4,5-dione and glutathione conjugate could not be blocked by fluoxetine (10 or 100 μM) or by a glutathione transferase inhibitor, boric acid/serine. The results indicate a different molecular mechanism among 5-HT derived neurotoxins and suggest that tryptamine-4,5-dione and/or its glutathione conjugate would cause neuronal damage, if they are formed in vivo.

Keywords: Tryptamine-4,5-dione; 5-HT (5-hydroxytryptamine, serotonin); Neurotoxicity; Free radical; Fenton

1. Introduction

Tryptamine-4,5-dione is formed by electrochemical oxidation of serotonin (5-HT) in vitro (Chen et al., 1989). Like the well-documented serotonin neurotoxins, 5,6- or 5,7-dihydroxytryptamine, tryptamine-4,5-dione has been shown to produce neurotoxic effects in the rodent brain (Wrona and Dryhurst, 1987). After intracerebroventricular administration of tryptamine-4,5-dione in the rat, terminal degeneration and neuronal argyrophilia were observed in various limbic structures, such as hippocampus, entorhinal cortex and cingulate cortex, while no cell degeneration occurred in the dorsal raphe nucleus (Crino et al., 1989). In addition, tryptamine-4,5-dione has been demonstrated to have a selective affinity, in membrane preparation, for the GTP binding site of Go and Gi protein (Fishman et al., 1991). Besides being produced by electrolysis from an acidic 5-HT solution, tryptamine-4,5-dione can also be formed by benzeneselenic anhydride oxidation of 5-HT (Cai et al., 1990), or by enzymatic oxidation of 5-HT at physiological pH (Wrona and Dryhurst, 1991). These data suggest that tryptamine-4,5-dione may also be formed in vivo, possibly by an oxidative enzyme or potent oxidizing agent, such as oxygen free radicals. If tryptamine-4,5-dione is formed via a free-radical reaction in then central nervous system, it could participate in the pathological process of neuronal damage induced by ischemia or traumatic brain injury (Ikeda et al., 1989; Sakamoto et al., 1991). In this study, we endeavored to (1) demonstrate the formation of tryptamine-4,5-dione in the presence of 5-HT by two well-defined free-radical-generating systems, i.e. xanthine oxidase and the Fenton reaction and; (2) develop a brain neuronal culture system to study the molecular mechanism of tryptamine-4,5-dione mediated neurotoxicity and compare its toxic effect with the 5,6- and 5,7-dihydroxytryptamine neurotoxins.

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2. Materials and methods

2.1. Xanthine oxidase reaction

Conversion of hypoxanthine to xanthine and uric acid, catalyzed by xanthine oxidase, results in the production of superoxide ($^{\cdot}O2^{-}$) and hydroxyl radical ($^{\cdot}OH$) (Kuppusamy and Zweier, 1989). To investigate oxidation of 5-HT by oxygen free radicals, 0.1 mM 5-HT HCl (Sigma, St. Louis, MO, USA) was incubated in 0.1 M phosphate buffer (pH 7.0) with 0.1 U xanthine oxidase, 0.1 mM $FeSO_4$, 0.3 mM EDTA and 0.1 mM hypoxanthine at room temperature for various time intervals. A control reaction was performed similarly in the absence of hypoxanthine and/or $FeSO_4$. The reaction was terminated by adding 0.2 N perchloric acid and the reaction mixture was centrifuged at 15000 × g for 15 min. Since tryptamine-4,5-dione has a high affinity for sulfhydryl residues, such as reduced glutathione (Cai et al., 1990; Wong et al., 1993), we also combined 2–10 μM of reduced glutathione and an equal amount of perchloric acid extract in a phosphate buffer to obtain an additional chromatographic marker for tryptamine-4,5-dione formation. Perchloric acid extract and reaction mixtures were analyzed by the Coulometric electrode array system (CEAS model 55-0650, ESA, Bedford, MA, USA) (Chen et al., 1992).

2.2. Fenton reaction

Oxidation of 5-HT by hydroxyl radicals was studied using the Fenton reaction which generates $^{\cdot}OH$ from hydrogen peroxide and reduced iron (Sutton and Winterbourn, 1989). In these experiments, 0.1 mM 5-HT was incubated in a phosphate buffer (0.1 M, pH 3–6) with varying concentrations of H_2O_2 and $FeSO_4$ at 22°C for 180 min. The reaction products were analyzed by CEAS (55-0650 system).

2.3. Electrochemical preparation of tryptamine-4,5-dione

Preparation of tryptamine-4,5-dione from 5-HT in vitro was performed in a fritt-type electrochemical synthesis cell (ESA) as described previously (Chen et al., 1989). In brief, counter and reference electrodes were filled with 0.5 N HCl and 1.0 M NaCl solutions respectively and immersed in a Teflon cylinder which contained a solution of 0.1 mM 5-HT in 0.01 N HCl. The solution flowed by gravity through a porous carbon working electrode which was maintained at a constant potential of +670 mV by a coulometric potentiostat (Coulochem 5100, ESA). Usually, a greater than 90% yield of tryptamine-4,5-dione was achieved as monitored by its unique voltammogram in CEAS system. Tryptamine-4,5-dione was concentrated over an Amberlite ion-exchange column to a maximum concentration of 1 mM. The tryptamine-4,5-dione solution was diluted to 1–100 μM with culture medium for the subsequent toxicity tests. Inactive tryptamine-4,5-dione was prepared by allowing complete degradation of the tryptamine-4,5-dione by exposure to room air which produced an electrochemically inactive brown compound. The ethylcarbonate derivative of tryptamine-4,5-dione was prepared as described (Cai et al., 1990).

2.4. Coulometric electrode array system (CEAS)

A high performance liquid chromatography (HPLC) system with two C18 reverse-phase column (3 μm, HR-80, ESA) consisted of a Bio-Rad HPLC pump (model 1300) and a sample injector (Rheodyne 7125). The electrochemical detector was an array of 16 on-line coulometric detector cells with applied potentials from 0 to +900 mV in increments of 60 mV. The mobile phase consisted of an organic and ion-pair gradient with a flow rate of 1 ml/min. It contained 0.1 M NaH_2PO_4, 50 μl/l tetrahydrofuran and 10 nM nitrilotriacetic acid with pH 3.35. Mobile phase A contained 50% methanol and 50 mg/l sodium dodecyl sulfate. Mobile phase B contained no methanol and 10 mg/l sodium dodecyl sulfate.

2.5. Cultures of embryonic chick brain neurons and viability assay

The cell culture of 9-day chicken embryo forebrain neurons was prepared as described (Aizenman et al., 1986). This preparation consists of > 95% neurons as determined by staining with antibodies specific for neurofilaments or glial fibrillary protein (data not shown). The survival assay was performed on polylysine coated 96 well ELISA plates essentially as described (Mossman, 1983). To each well, 4×10^4 neurons in 100 μl of chemically defined culture medium (1 : 1 F12, DMEM, Sigma) containing 100 μg/ml chicken apotransferrin and 5 μg/ml bovine insulin were added. Different concentrations of toxins or buffer controls were added to each row of six wells. At various time thereafter, 50 μg of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in 10 μl of buffer were added to each well. After 4 h at 37°C the resulting blue precipitate, formed by mitochondrial reduction in living cells, was dissolved by the addition of acidic isopropanol and the optical density read in an automated ELISA plate reader. The assay was linear in the range of cell survival obtained here.

2.6. Chemicals

Chemicals were obtained from Sigma Chem. (St. Louis, MO, USA) except for phosphoric acid, methanol and *l*-decane sulfonic acid which were purchased from Aldrich Chem., Milwaukee, WI, USA. Fluoxetine was a generous gift from Eli Lilly Co. (Indiana, USA).

2.7. Data analysis

All data in this report are expressed as means ± S.E.M. A non-paired *t*-test was applied to analyze differences

between control and toxin-treated groups in viability assay. One-way analysis of variance (ANOVA), followed by a post hoc Scheffé comparison was used to analyze the differences between toxin and various drug treatment, and among different incubation time of toxin-treated groups. A $P < 0.05$ was considered significant.

3. Results

3.1. Formation of tryptamine-4,5-dione from 5-HT by xanthine oxidase-hypoxanthine and by the Fenton reaction

A significant product which appeared when 5-HT was incubated with the xanthine oxidase-hypoxanthine system had identical chromatograph (retention time = 9.5 min) and voltammogram as tryptamine-4,5-dione prepared by electrochemical oxidation of 5-HT (Fig. 1). As was described with tryptamine-4,5-dione (Chen et al., 1989), this product had a high affinity for reduced glutathione and the conjugate had identical electrochemical properties as glutathione conjugated to tryptamine-4,5-dione prepared by electrochemical oxidation (Fig. 1). The optimal pH for production of tryptamine-4,5-dione using the xanthine oxidase-hypoxanthine system was pH 7.0, and a 7.4 μM concentration was reached after 1 h of incubation. The concentration of tryptamine-4,5-dione was much lower at pH 6.0 (2.6 μM) or pH 8.0 (0.8 μM), and barely detectable at pH 4.0 or 5.0. The loss of 5-HT was greatest at pH 7.0, at which the yield of tryptamine-4,5-dione during the incubation gradually increased during the first hour, in parallel with the loss of 5-HT (Fig. 2). However, only a portion of 5-HT was recovered as tryptamine-4,5-dione. No signal corresponding to 5-hydroxyindoleacetic acid, a major oxidative metabolite in vivo was observed. There

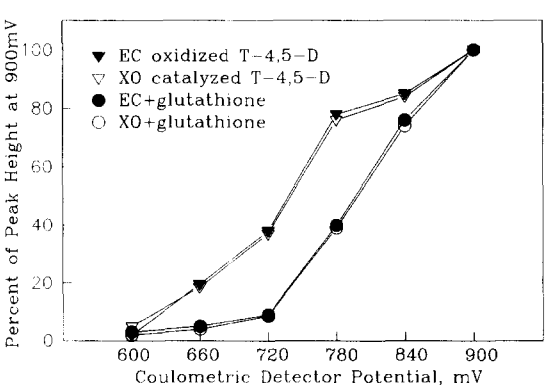

Fig. 1. Voltammograms of tryptamine-4,5-dione (T-4,5-D) and T-4,5-D + glutathione conjugate. Electrochemically (EC) synthesized T-4,5-D and its glutathione conjugate were compared with xanthine oxidase (XO)-hypoxanthine catalyzed formation of T-4,5-D and its glutathione conjugate. Voltammograms were generated by a 16-channel CEAS system. The oxidation current (exhibited as peak height in the chromatogram) at a specific potential was expressed as percentage to the value obtained at 900 mV.

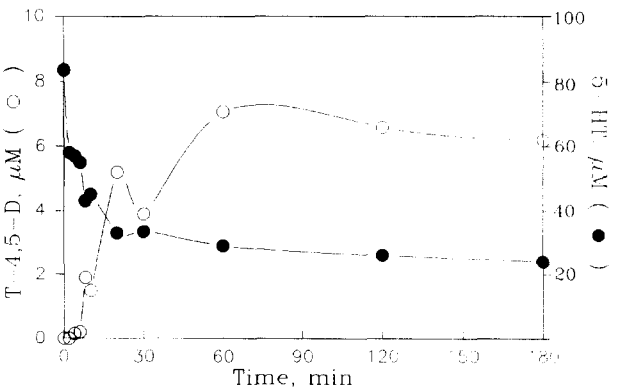

Fig. 2. Time-course of formation of T-4,5-D from 5-HT during incubation with xanthine oxidase-hypoxanthine at pH 7.0 and room temperature. The initial concentration of 5-HT was 100 μM and the graph indicates the concentrations of 5-HT remaining at the end of the incubation period.

was also no signal which would correspond to 5,6- or 5,7-dihydroxytryptamine. In the absence of $FeSO_4$ and/or hypoxanthine in the xanthine oxidase reaction, no tryptamine-4,5-dione signal was detected.

Incubation of 0.1 mM 5-HT in the presence of hydrogen peroxide and reduced iron yielded a product which had the same electrochemical and chromatographic properties as tryptamine-4,5-dione prepared by electrochemical oxidation and/or the hypoxanthine-xanthine oxidase reaction (data not shown). The rate of tryptamine-4,5-dione formation by the Fenton reaction was pH dependent and decreased with increasing pH. The highest yield of tryptamine-4,5-dione was obtained at pH 3.0 (4.4 μM). At that pH, yields of tryptamine-4,5-dione were dependent on the concentrations of $FeSO_4$ and H_2O_2. Usually, a maximum of 1–5 μM tryptamine-4,5-dione was observed when $FeSO_4$ and H_2O_2 concentrations were within the limit of 1–10 μM and 1–100 μM, respectively. No tryptamine-4,5-dione signal was observed by CEAS when $FeSO_4$ or H_2O_2 concentrations were above or below that range.

3.2. Neurotoxicity of tryptamine-4,5-dione, 5,6- and 5,7-dihydroxytryptamines on cultured embryonic chick brain neurons

The effect of electrochemically synthesized tryptamine-4,5-dione on the survival of embryonic chick brain neurons cultured in a defined, serum-free medium is shown in Fig. 3A. There was a dose-dependent relationship of tryptamine-4,5-dione neurotoxicity on cultured neurons in which 100 μM tryptamine-4,5-dione caused a 68% decrease in surviving neurons. At 10 μM a 25% loss was seen and at 1 μM or lower concentrations, no significant decrease in surviving neurons was detected. 5-HT or inactive tryptamine-4,5-dione at a concentration of 100 μM had no effect on neuronal survival. As can be seen in Fig. 3B, the decreased neuronal survival is apparent after one day of incubation with tryptamine-4,5-dione and remained

relatively constant for the next three days. The effect of more stable derivative of tryptamine-4,5-dione, the ethyl carbonate derivative (Cai et al., 1990), however, showed a time-dependent neurotoxicity increasing for up to four days.

The neurotoxicity of tryptamine-4,5-dione, 5,6- and 5,7-dihydroxytryptamines on cultured chick neurons is compared in Fig. 4. A significant decrease in neuronal survival was observed with 5,6- and 5,7-dihydroxytryptamines. The addition of the sulfhydryl compound, glutathione at 2 or 10 μM significantly enhanced the toxicity induced by 10 μM tryptamine-4,5-dione (Fig. 4A). In addition, combination of 10 μM glutathione with 1 μM tryptamine-4,5-dione could induce the neurotoxicity that could not be observed when 1 μM tryptamine-4,5-dione was added alone (with glutathione, 66% survival rate; without, 84% survival rate; data not shown). On the contrary, glutathione at 2 μM partially recovered while at 10 μM totally prevented the neurotoxic effect caused by 10 μM 5,6- and 5,7-dihydroxytryptamines in the cultured neurons (Fig. 4B). The toxicity induced by 10 μM 5,6- or 5,7-dihydroxytryptamine was fully prevented by the 5-HT uptake inhibitor, fluoxetine at 10 μM. However, the enhanced toxicity resulted from co-administration of 10 μM tryptamine-4,5-dione and glutathione could not be blocked by either 10 or 100 μM fluoxetine or by a glutathione transferase inhibitor, boric acid/serine at 10 μM (Meister and Anderson, 1983). Incubation of 10 μM glutathione in the presence or absence of 10 μM 5-HT had no effect on neuronal viability.

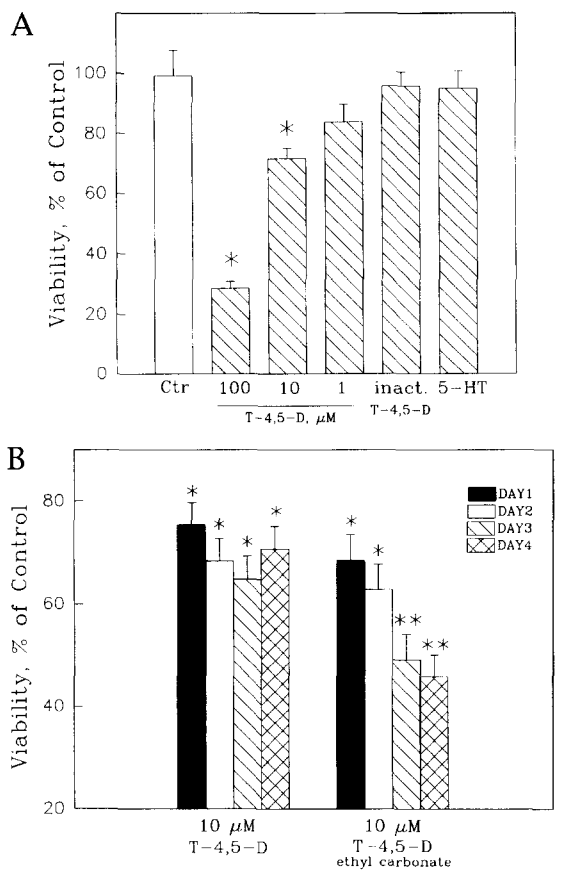

Fig. 3. Effects of concentration and incubation time of T-4,5-D and T-4,5-D ethyl carbonate on neuron survival in vitro. 4×10^4 dissociated 9-day-old chick embryo neurons were plated on each well of 96-well ELISA plate. (A) Various concentrations of T-4,5-D and 100 μM of inactive T-4,5-D and 5-HT or buffer controls were added to each row of six wells immediately after plating. The MTT viability assay was performed 96 h later. (B) 10 μM of T-4,5-D or its ethyl carbonate derivative was added to the wells at day 0. MTT viability assay was performed at various time thereafter. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with 10 μM T-4,5-D ethyl carbonate at day 1.

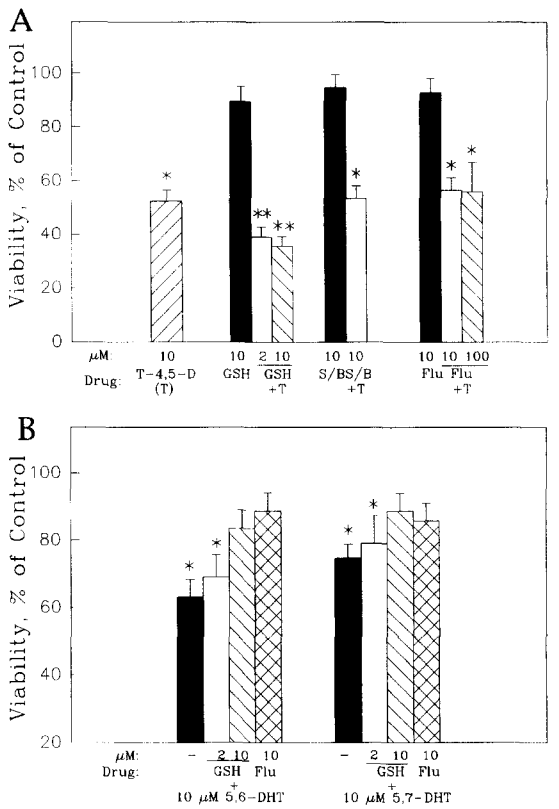

Fig. 4. Effects of 2 and 10 μM glutathione and drugs on T-4,5-D, 5,6- and 5,7-dihydroxytryptamines (5,6- and 5,7-DHT) caused neurotoxicity. Culturing and viability assay were performed as described in Fig. 3. (A) Neurotoxicity of electrochemically synthesized T-4,5-D (T) in the presence or absence of 2 and 10 μM glutathione (GSH), 10 μM serine/boric acid (S/B) or 10 and 100 μM fluoxetine (Flu) in the cultured neurons. (B) Neurotoxicity of 5,6-DHT and 5,7-DHT in the presence of 2 and 10 μM glutathione or 10 μM fluoxetine. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with 10 mM T-4,5-D.

4. Discussion

This study demonstrates that (1) the formation of neurotoxic tryptamine-4,5-dione can be catalyzed by exposure of 5-HT to simulated in vivo conditions, i.e. an oxygen free-radical-generating system of xanthine-hypoxanthine or to the Fenton reaction; (2) tryptamine-4,5-dione has neuro-

toxic effects on cultured chick neurons which is apparently different from the two well-established 5-HT derived neurotoxins, 5,6- and 5,7-dihydroxytryptamines.

Oxidation of 5-HT is a complex process, resulting in the formation of many compounds (for review, see Dryhurst, 1990). In this report, we concentrate on the formation of tryptamine-4,5-dione, a compound which is toxic when intraventricularly administered into mice brain (Wrona and Dryhurst, 1987), and in rats the compound has unique neurotoxic properties that selectively damage the limbic structures (Crino et al., 1989). The formation of tryptamine-4,5-dione was also observed after incubation of 5-HT with peroxidase, ceruloplasmin or tyrosinase (Wrona and Dryhurst, 1990). Although dimers and trimers of 5-HT are also formed during these reactions, several of them are ultimately hydrolyzed and yield tryptamine-4,5-dione. Thus this compound might represent a common (if not the major) oxidative product of 5-HT through various enzymatic or non-enzymatic catabolic pathways.

We endeavored to test the possibility that tryptamine-4,5-dione could also be formed from 5-HT by oxygen free-radical interaction. When 5-HT was incubated with xanthine oxidase and hypoxanthine in the presence of Fe^{2+}-EDTA, a tryptamine-4,5-dione signal did appear in the solution which depended on the consumption of 5-HT. A large amount of 5-HT was not converted into tryptamine-4,5-dione by xanthine oxidase-hypoxanthine, possibly due to the formation of electrochemical unresponsive compound(s) or polymer(s) since inactive tryptamine-4,5-dione could not be detected by CEAS. Hypoxanthine and xanthine oxidase are normally present in the brain at very low concentrations (Schultz and Lowenstein, 1976). Under some pathological conditions, e.g. ischemia, hypoxanthine is formed from ATP, and xanthine oxidase activity is increased by a conversion of xanthine dehydrogenase to xanthine oxidase (Kinuta et al., 1989). The resulting high level of hypoxanthine and xanthine oxidase could produce several reactive oxygen species, including superoxide and hydroxyl free radicals that might facilitate oxidation reactions when the nearby 5-HT concentration is high. Thus, substances such as tryptamine-4,5-dione may be formed under those particular conditions.

Another source of oxygen free radicals in vivo could be hydrogen peroxide produced by monoamine oxidase (Cohen, 1986). Hydrogen peroxide serves as a substrate for the Fenton reaction which would proceed when free iron becomes available. This could occur during brain trauma that will result in an increased formation of free radicals and high levels of free iron (Chan et al., 1984). Previous evidence already indicated that under appropriate conditions, the Fenton reaction could occur in vivo and lead to DNA damage and mutation (Aruoma et al., 1989; Imlay et al., 1988). At present, we demonstrated that in vitro tryptamine-4,5-dione could be formed by a Fenton reaction, and it is possible that this reaction could occur in vivo considering the high levels of 5-HT in brain serotonergic terminals that contain large amounts of monoamine oxidase. The variable yield of tryptamine-4,5-dione in the current experiment reflected the chemical complexity of free-radical and indoleamine interactions (Dryhurst, 1990). When reduced glutathione was added into the reaction mixture, the signal in the chromatogram shifted. The new compound appeared to be 7-*S*-glutathionyl-tryptamine-4,5-dione, a conjugate of glutathione and tryptamine-4,5-dione which has been identified previously in this laboratory (Cai et al., 1990) and by Wong et al. (1993). Since this compound is relatively stable as compared with parent tryptamine-4,5-dione, it is conceivable that the conjugate could be used as a valid molecular marker for detection of whether altered 5-HT metabolism occurred in CNS under pathological conditions, such as oxidative stress.

A dose-dependent neurotoxicity of tryptamine-4,5-dione on cultured embryonic chick brain neurons was demonstrated in a serum-free medium. The electrochemically synthesized tryptamine-4,5-dione acted rapidly to destroy neurons and its toxic effect only persisted for 24 h. This is not unexpected given the instability of tryptamine-4,5-dione in solution (Wrona and Dryhurst, 1987). Inactive tryptamine-4,5-dione forms over time, presumably resulting from polymerization of tryptamine-4,5-dione, and has no detectable toxic effect on chick neurons. This result is consistent with results previously obtained in vivo (Crino et al., 1989). When the more stable ethyl carbonate tryptamine-4,5-dione derivative (Cai et al., 1990) was added to the culture medium, it progressively killed the neurons up to 96 h without forming a precipitate.

Since we have previously shown that in vitro tryptamine-4,5-dione can interact with the GTP-binding site of G-protein in a manner which is dependent on free sulfyhydryls (Fishman et al., 1991), we also tested the effect of the glutathione adduct on the toxicity of tryptamine-4,5-dione in the cultured neurons. The observed enhancement of neurotoxicity induced by glutathione and tryptamine-4,5-dione conjugate (presumably 7-*S*-glutathionyl-tryptamine-4,5-dione) at various combinations is consistent with recent results of Dryhurst and coworkers (Wong et al., 1993) showing that 7-*S*-glutathionyl-tryptamine-4,5-dione is toxic when administered into mice brain. It also agrees with a previous report that conjugation of 1,4-benzoquinone with glutathione leads to intensified nephrotoxicity (Lau et al., 1988). The conjugate of 1,4-benzoquinone with glutathione was found to enter the cell via glutathione *S*-transferase and to release the toxic quinone chemical inside. We tested if 7-*S*-glutathionyl-tryptamine-4,5-dione mediated a similar mechanism by adding transferase inhibitor, boric acid/serine in the culture medium. Since high concentrations of boric acid/serine (> 10 μM) could cause neuronal death, we examined its biological effect at a maximal dose of 10 μM. This treatment, however, failed to block the enhanced toxicity of 7-*S*-glutathionyl-tryptamine-4,5-dione. Considering that 7-*S*-glutathionyl-tryptamine-4,5-di-

one could further react with excess of GSH and participate in series of redox reactions that generate toxic H_2O_2 (Wong et al., 1993), it is highly likely that the inhibition of intracellular tryptamine-4,5-dione formation can not protect against the extracellular damage caused by the 7-*S*-glutathionyl-tryptamine-4,5-dione redox reaction.

Additionally, both 5,6- and 5,7-dihydroxytryptamines cause a significant neurotoxicity on cultured chick neurons. It has been known that the quinone product of 6-hydroxydopamine and 5,6-tryptamine, mediated by an oxygen dependent reaction, are the major reactive species affecting the -SH binding of cellular protein and eventually cause cell death (Rotman et al., 1976). The toxic effect of 5,6- and 5,7-dihydroxytryptamines, however, can be prevented by glutathione as well as by the 5-HT uptake inhibitor, fluoxetine. These results indicate that the essential toxic species of 5,6- and 5,7-dihydroxytryptamines are quinone structures, and the selectivity of their toxic effect on 5-HT neurons is determined by a high affinity, fluoxetine-sensitive 5-HT uptake mechanism. Although the molecular target(s) for tryptamine-4,5-dione could not be identified yet, the current findings that glutathione and fluoxetine had no effect on the tryptamine-4,5-dione-mediated neurotoxicity tend to distinguish the mode of action of 5,6- and 5,7-dihydroxytryptamines from that of tryptamine-4,5-dione.

These data suggest that an exposure of 5-HT to oxygen-derived free-radical-generating systems at physiological pH results in formation of the neurotoxin, tryptamine-4,5-dione at a concentration that was found to have neurotoxic properties in vivo and in vitro. In addition, the molecular mechanism of tryptamine-4,5-dione neurotoxicity is apparently different from that of 5,6- and 5,7-dihydroxytryptamines. The current findings along with others (Wrona and Dryhurst, 1988) suggest that tryptamine-4,5-dione and/or its GSH conjugate, 7-*S*-glutathionyl-tryptamine-4,5-dione would impair neuronal survival, if they were formed in vivo.

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