Tryptamine-4,5-dione, a Putative Endotoxic Metabolite of the Superoxide-Mediated Oxidation of Serotonin, Is a Mitochondrial Toxin: Possible Implications in Neurodegenerative Brain Disorders

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The release and subsequent reuptake of 5-hydroxytryptamine (5-HT) and cytoplasmic superoxide ($O_2^{-\bullet}$) generation have both been implicated as important factors associated with the degeneration of serotonergic neurons evoked by methamphetamine (MA) and cerebral ischemia-reperfusion (I-R). Such observations raise the possibility that tryptamine-4,5-dione (T-4,5-D), the major in vitro product of the $O_2^{-\bullet}$-mediated oxidation of 5-HT, might be an endotoxicant that contributes to serotonergic neurodegeneration. When incubated with intact rat brain mitochondria, T-4,5-D (≤100 μM) uncouples respiration and inhibits state 3. Experiments with rat brain mitochondrial membrane preparations confirm that T-4,5-D evokes irreversible inhibition of NADH-coenzyme Q_1 (CoQ_1) reductase and cytochrome *c* oxidase (COX) apparently by covalently modifying key sulfhydryl (SH) residues at or close to the active sites of these respiratory enzyme complexes. Ascorbic acid blocks the inhibition of NADH-CoQ_1 reductase by maintaining T-4,5-D predominantly as 4,5-dihydroxytryptamine (4,5-DHT), thus preventing its reaction with SH residues. In contrast, ascorbic acid potentiates the irreversible inhibition of COX by T-4,5-D. This may be because the T-4,5-D−4,5-DHT couple redox cycles in the presence of excess ascorbate and molecular oxygen to cogenerate $O_2^{-\bullet}$ and H_2O_2 that together react with trace levels of iron to form an oxo−iron complex that selectively damages COX. Thus, T-4,5-D might be an endotoxicant that, dependent on intraneuronal conditions, mediates irreversible damage to mitochondrial respiratory enzyme complexes and contributes to the serotonergic neurodegeneration evoked by MA and I-R.

An elevated level of production of reactive oxygen species (ROS[1]), a condition known as oxidative stress (*1*), appears to be an important factor in the pathological mechanisms that underlie many neurodegenerative brain disorders (*2*) such as Alzheimer's disease (AD) (*3*) and Parkinson's disease (PD) (*4*) and the neurodegeneration evoked by cerebral ischemia-reperfusion (I-R) (*5*) and methamphetamine (MA) (*6*). Indeed, by causing oxidative damage to lipids, proteins, and nucleic acids (*7*), ROS have been proposed to directly mediate the neurodegeneration in AD (*3*) and PD (*4*). However, a considerable body of evidence also suggests that defects in mitochondrial (mt) energy metabolism are also important pathological factors in neurodegenerative brain disorders (*2*, *8*), although a clear connection between oxidative stress and such defects has not been established.

Several lines of evidence suggest that the roles of ROS in neurodegenerative brain disorders might be more complex than simply oxidation of important macromolecules. This evidence is drawn primarily from studies of the neurodegeneration evoked by MA and I-R using experimental animals. MA (*6*, *9*) and I-R (*10*, *11*) evoke the degeneration of serotonergic, dopaminergic, and other neurons in various brain regions. The neurotoxic properties of MA are not caused directly by the drug itself or its known metabolites (*6*, *9*). Recent evidence suggests that the initial step in MA-induced neurotoxicity is a severe perturbation of energy metabolism (*12*, *13*) and rapid ATP depletion (*14*) with neuronal depolarization (*12*) that evokes a massive release of 5-hydroxytryptamine (5-HT) (*15*) and dopamine (DA) (*16*) and a delayed but prolonged elevation of extracellular concentrations of glutamate (Glu) (*17*). Ischemia similarly causes a rapid loss of brain ATP (*18*), neuronal depolarization, and release of 5-HT (*19*), DA (*20*), and other neurotransmitters, including Glu (*20*, *21*). Elevation of extracellular concentrations of Glu, particularly under conditions of a reduced ATP production level, with resultant *N*-methyl-D-aspartate (NMDA) receptor activation can evoke excitotoxic damage (*22*, *23*) that is associated with superoxide ($O_2^{-\bullet}$) generation by neurons (*24*). The possibility that

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[1] Abbreviations: ROS, reactive oxygen species; AD, Alzheimer's disease; PD, Parkinson's disease; I-R, ischemia-reperfusion; MA, methamphetamine; mt, mitochondrial; 5-HT, 5-hydroxytryptamine; DA, dopamine; Glu, glutamate; NMDA, *N*-methyl-D-aspartate; $O_2^{-\bullet}$, superoxide radical anion; SOD, superoxide dismutase; CoQ_1, coenzyme Q_1; COX, cytochrome *c* oxidase; T-4,5-D, tryptamine-4,5-dione; CySH, L-cysteine; GSH, glutathione; NADH, nicotinamide adenine dinucleotide; BSA, bovine serum albumin; SOS, sodium octyl sulfate; KCN, potassium cyanide; RCR, respiratory control ratio; SH, sulfhydryl group; 7-*S*-Glu-4,5-DHT, 7-*S*-glutathionyl-4,5-dihydroxytryptamine; 7-*S*-Glu-T-4,5-D, 7-*S*-glutathionyltryptamine-4,5-dione; 4,5-DHT, 4,5-dihydroxytryptamine; HO•, hydroxyl radical; 5-HEO, 5-hydroxy-3-(ethylamino)-2-oxindole.

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intraneuronal $O_2^{-\bullet}$ is a key component of the mechanisms that evoke damage to serotonergic, dopaminergic, and other neurons is supported by the resistance of transgenic mice that overexpress cytoplasmic Cu/Zn superoxide dismutase (SOD) to the neurotoxic effects of MA (*25*, *26*) and I-R (*27*). Furthermore, NMDA receptor antagonists protect against MA- and I-R-mediated neuronal damage (*28*−*30*). However, while Glu can cause excitotoxicity primarily mediated by NMDA receptor activation (*22*, *23*), elevation of extracellular concentrations of Glu by itself may not be sufficient to cause neuronal damage (*31*, *32*). It is interesting, therefore, that both the uptake and intraneuronal oxidation of monoamine neurotransmitters present in serum are essential for Glu oxidative toxicity (*33*). Furthermore, neurons cultured in serum exhibit excitotoxic damage when exposed to Glu but fail to respond to high concentrations of this excitotoxin when cultured under serum-free conditions (*34*), implying, perhaps, that monoamine neurotransmitters may be involved in NMDA receptor-mediated excitotoxicity. Indeed, 5-HT and DA are key participants in the neurotoxic mechanisms evoked by MA and I-R because their uptake and synthesis inhibitors are neuroprotective (*35*−*38*).

Neuronal death resulting from cerebral ischemia occurs during reperfusion (reoxygenation) and develops over a period of hours or days, depending on brain region (*11*), and coincides with defects in mt respiration principally involving decreased complex I [NADH-coenzyme Q (CoQ) reductase], complex IV [cytochrome *c* oxidase (COX)], and pyruvate dehydrogenase complex activities (*39*−*42*). Significant decreases of COX activity also occur in many brain regions following MA administration (*43*).

The preceding information raises the possibility that the release and subsequent reuptake of 5-HT and DA and their intraneuronal oxidation by $O_2^{-\bullet}$, generated as a result of NMDA receptor activation by elevated extracellular concentrations of Glu under conditions of neuronal energy impairment evoked by MA or I-R, might lead to formation of mt toxicants. Indeed, both 5-HT (*44*) and DA (*45*) are readily oxidized by $O_2^{-\bullet}$. The major product of the in vitro oxidation of 5-HT by $O_2^{-\bullet}$ is tryptamine-4,5-dione (T-4,5-D) (*44*). This paper describes the influence of T-4,5-D on rat brain mt respiration and on the activities of NADH−CoQ_1 reductase and COX.

Materials and Methods

Chemicals. 5-HT (creatinine sulfate), L-cysteine (CySH), cytochrome *c* (from bovine heart), glutathione (GSH), ascorbic acid, nicotinamide adenine dinucleotide (NADH), adenosine 5′-diphosphate (ADP), L-(−)-malic acid (disodium salt), succinic acid (disodium salt), pyruvic acid (sodium salt), glycylglycine, ethylenediaminetetraacetic acid (dipotassium salt, K_2EDTA), Trizma hydrochloride, bovine serum albumin (BSA, fatty acid free), citric acid monohydrate, sodium octyl sulfate (SOS), sucrose, phospholipid (asolectin from soybean), catalase, SOD, mannitol, and potassium cyanide (KCN) were obtained from Sigma (St. Louis, MO). Rotenone was obtained from Aldrich (Milwaukee, WI). All commercial chemicals were of the highest quality available and were used without further purification. Coenzyme Q_1 (ubiquinone-1) was synthesized according to the method of Naruta and Maruyama (*46*). Procedures for synthesizing T-4,5-D (*44*) and 7-*S*-glutathionyltryptamine-4,5-dione (7-*S*-Glu-T-4,5-D) (*47*) have been described elsewhere.

Rat Brain Mitochondrial Preparations. Mitochondria were prepared from the brains of male albino Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) as described previously (*48*). The final mt pellet for oxygen consumption studies and NADH-CoQ_1 reductase activity measurements was suspended in 300−350 μL of medium A [300 mM mannitol, 15.1 mM Trizma hydrochloride, 15 mM glycylglycine, and 0.1 mM K_2EDTA (pH 7.4)]. For oxygen consumption experiments in which an oxygen electrode assembly was used, freshly prepared intact mitochondria were stored on ice. For NADH-CoQ_1 reductase assays, mt preparations were stored at −80 °C until they were needed. For COX assays, the final mt pellet was suspended in medium B [225 mM mannitol, 75 mM sucrose, and 0.2 mM K_2EDTA (pH 7.4)] and stored at −25 °C until it was needed. Protein was determined by the method of Lowry et al. (*49*).

Oxygen Electrode Studies. The extent of oxygen consumption was measured using a YSI (Yellow Springs Instrument Co., Yellow Springs, OH) model 5300 biological oxygen monitor equipped with a model 5357 micro oxygen probe assembly thermostated at 30 °C. Mitochondria (final concentration of 200−400 μg/mL protein) were suspended in 600 μL of air-saturated medium C [300 mM mannitol, 10 mM Trizma hydrochloride, 10 mM KCl, 5 mM potassium phosphate, 0.1 mM K_2EDTA, and 1 mg/mL BSA (pH 7.2)]. T-4,5-D (HCl salt) was dissolved in freshly prepared medium C immediately prior to addition of mitochondria. These preparations were introduced into the oxygen electrode chamber and incubated for predetermined times at 30 °C, and then 2 μL of pyruvate with malate (each at 0.75 M to give a final concentration of 2.5 mM) was added. State 3 respiration was initiated by addition of 2 μL of 0.135 M ADP (final concentration of 0.45 mM). To determine time-dependent effects on oxygen consumption, mitochondria were added to medium C containing T-4,5-D and incubated for up to 10 min prior to addition of malate and pyruvate and then ADP. The rate of state 3 oxygen consumption was determined from the linear segment of the oxygen concentration versus time trace after addition of ADP. The rate of state 4 oxygen consumption was determined from the oxygen consumption rate measured prior to addition of ADP. The effect of T-4,5-D on complex II respiration was determined by adding 4 μL of 0.75 M succinate (final concentration of 5 mM).

NADH-CoQ_1 Reductase Assays. Mitochondria were exposed to six freeze−thaw cycles immediately prior to assays to ensure maximal NADH-CoQ_1 reductase activity. NADH-CoQ_1 reductase activities were measured using a Beckman DU640 spectrophotometer. Rat brain mt membranes were 93−95% rotenone-sensitive in NADH-CoQ_1 reductase assays.

(1) Method I. This method was employed to follow the time course of NADH-CoQ_1 reductase inhibition by T-4,5-D. Frozen−thawed rat brain mitochondria (400 μg of mt protein in medium A) were incubated with (experimental) or without (control) T-4,5-D in a total volume of 200 μL of 20 mM potassium phosphate buffer (pH 8) at 30 °C for times ranging from 0 to 90 min. Then, 20 μL of the resulting solution (containing 40 μg of mt protein) was transferred into the assay solution consisting of 870 μL of 20 mM potassium phosphate buffer (pH 8), 50 μL of asolectin [15 mg/mL in 20 mM phosphate buffer (pH 8.0) containing 1 mM K_2EDTA], 50 μL of 30 mM KCN (in 20 mM phosphate buffer), and 50 μL of 1.0 mM CoQ_1 (in 10% ethanol/water, v/v) contained in a quartz UV cell (0.5 cm optical path length). This solution was incubated for 5 min at 30 °C, and then 25 μL of 10 mM NADH (in water) was added to initiate the reaction. The activity of NADH-CoQ_1 reductase was measured by monitoring the decrease in the NADH concentration at 340 nm using a molar absorption coefficient of 6.22 mM^{-1} cm^{-1}.

(2) Method II. This method was employed to investigate the irreversible inhibition of NADH-CoQ_1 reductase by T-4,5-D. Frozen−thawed rat brain mitochondria (200 μg of mt protein) were incubated with T-4,5-D in 100 μL of 20 mM potassium phosphate buffer (pH 8) for 60 min at 30 °C. In some experiments, GSH, CySH, ascorbic acid, catalase, or SOD was included in this initial incubation solution. The mt membranes were then washed by adding 500 μL of 20 mM potassium phosphate buffer (pH 8) and centrifuged at 150000*g* and 4 °C for 10 min (Beckman L8-80 centrifuge with a SW65 rotor). The pellet was gently

Table 1. Effects of T-4,5-D on State 4, State 3 (Complex I), and Complex II Mitochondrial Oxygen Consumption

T-4,5-D concentration (μM)	rate of oxygen uptake[a,b] (ng of atoms of oxygen min^{-1} mg of protein^{-1}) state 4	state 3 (complex I)	respiratory control ratio	complex II
5 min Initial Incubation Time with T-4,5-D				
0 ($n = 17$)	21.46 ± 2.07	111.35 ± 6.60	5.51 ± 0.25	68.37 ± 6.03
10 ($n = 6$)	21.62 ± 4.98	83.53 ± 13.51	4.22 ± 0.41[d]	70.84 ± 12.34
20 ($n = 6$)	26.31 ± 3.14	95.74 ± 9.95	3.73 ± 0.29[e]	84.44 ± 4.64
30 ($n = 8$)	36.08 ± 4.54[e]	80.31 ± 7.92[d]	2.28 ± 0.15[f]	80.95 ± 2.94
50 ($n = 7$)	43.64 ± 7.54[e]	61.11 ± 10.04[f]	1.46 ± 0.15[f]	60.63 ± 14.15
100 ($n = 7$)	47.99 ± 6.44[f]	[c]	1.0	[c]
200 ($n = 3$)	41.77 ± 9.30[e]	[c]	1.0	[c]
500 ($n = 6$)	14.86 ± 2.39	[c]	1.0	[c]
10 min Initial Incubation Time with T-4,5-D				
0 ($n = 7$)	23.37 ± 2.34	107.83 ± 11.36	4.64 ± 0.22	65.97 ± 6.36
10 ($n = 5$)	20.77 ± 2.52	67.56 ± 8.14[d]	3.26 ± 0.10[e]	54.22 ± 7.36
20 ($n = 6$)	31.58 ± 4.03	71.76 ± 5.38[d]	2.39 ± 0.27[f]	71.51 ± 5.26
30 ($n = 4$)	46.26 ± 5.63[e]	60.37 ± 6.70[d]	1.31 ± 0.03[f]	86.88 ± 7.17
50 ($n = 6$)	42.27 ± 6.59[d]	43.73 ± 6.36[e]	1.04 ± 0.02[f]	54.10 ± 8.71
100 ($n = 4$)	47.29 ± 5.84[e]	[c]	1.0	[c]
200 ($n = 3$)	24.93 ± 6.76	[c]	1.0	[c]
500 ($n = 3$)	12.43 ± 1.91[d]	[c]	1.0	[c]

[a] Measured with a Clark-type oxygen electrode assembly; rat brain mitochondria (200−400 μg of protein/mL) were incubated in 600 μL of medium B for 5 or 10 min prior to addition of malate with pyruvate (2.5 mM final concentration for each). The rate of state 4 respiration was measured for 2 min and then ADP (0.45 μM) added to stimulate state 3. [b] Data are means ± SEM. [c] Complete inhibition. [d] $p < 0.05$. [e] $p < 0.005$. [f] $p < 0.0005$.

resuspended in 100 μL of 20 mM potassium phosphate buffer (pH 8), and then a 25 μL aliquot (containing 50 μg of mt protein) was added to the usual assay solution to determine the NADH-CoQ_1 reductase activity.

Cytochrome *c* Oxidase Assays. Cytochrome *c* was dissolved in 10 mM potassium phosphate buffer (pH 7.4) and reduced by addition of potassium ascorbate. Excess ascorbate was removed by dialysis using Spectrapor (Los Angeles, CA) size 3 membrane tubing against 10 mM phosphate buffer (pH 7.4) for 18−24 h with three changes of buffer. The concentration of ferrocytochrome *c* was determined spectrophotometrically (550 nm) using a molar absorption coefficient of 18.5 mM^{-1} cm^{-1}. Rat brain mt preparations were 93−95% KCN-sensitive in COX assays.

(1) Method III. This method was used to follow the time course of COX inhibition by T-4,5-D. Frozen−thawed rat brain mitochondria (200 μg of mt protein in medium B) were incubated with (sample) or without (control) T-4,5-D in a total volume of 500 μL of 20 mM potassium phosphate buffer (pH 7.4) at 30 °C for times ranging from 0 to 100 min. Then, 20 μL of the resulting solution (containing 8 μg of mt protein) was transferred to a 1 cm path length UV cell containing 940 μL of 20 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by adding 45 μL of 233 μM ferrocytochrome *c* in 10 mM potassium phosphate buffer (final concentration of 10.4 μM). The activity of COX was measured by monitoring the decrease in ferrocytochrome *c* concentration in the assay mixture for 60 s at 550 nm (Beckman DU640 spectrophotometer).

(2) Method IV. This method was employed to investigate the irreversible inhibition of COX. Frozen−thawed rat brain mitochondria (200 μg of mt protein) were incubated with (sample) or without (control) T-4,5-D for 60 min at 30 °C in a total volume of 250 μL of 20 mM potassium phosphate buffer (pH 7.4). In some experiments, GSH, CySH, ascorbic acid, catalase, SOD, or mannitol was included in this incubation solution. The mt preparation was then washed by adding 400 μL of 20 mM phosphate buffer (pH 7.4) and centrifuged at 150000*g* and 4 °C for 10 min. The resulting pellet was suspended in 500 μL of 20 mM phosphate buffer (pH 7.4), and a 20 μL aliquot (containing 8 μg of mt protein) was added to the usual assay solution to determine the COX activity.

All enzyme activities were measured as nanomoles per minute per milligram of mt protein.

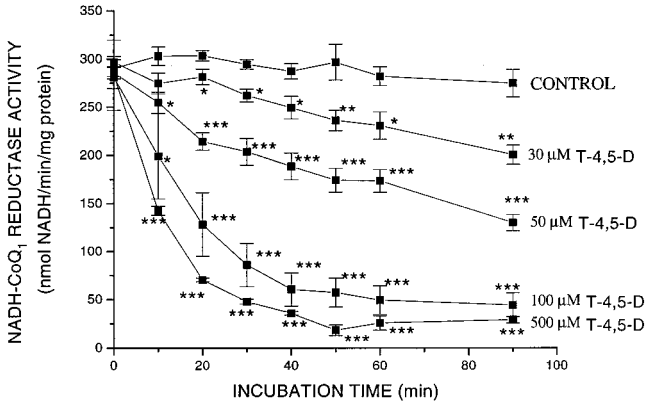


Figure 1. Time-dependent effects of T-4,5-D on NADH-CoQ_1 reductase activity. Frozen−thawed rat brain mitochondria (2 μg of protein/μL) were incubated in 20 mM potassium phosphate buffer (pH 8) in the absence (control) and presence of T-4,5-D at the specified concentrations. Aliquots were assayed at various times for NADH-CoQ_1 reductase activity (Method I in Materials and Methods). Data are means ± SEM (vertical bars) ($n \geq 3$). *$p < 0.05$. **$p < 0.005$. ***$p < 0.0005$.

Statistics. Results were obtained at least in triplicate and are presented as means ± SEM. A Student's *t* test was used to determine statistical significance with a *p* of <0.05 being taken to indicate a significant difference.

Results

Effects of T-4,5-D on Mitochondrial State 4 and State 3 Respiration. The effects of T-4,5-D on oxygen consumption by intact rat brain mitochondria stimulated by malate and pyruvate (state 4) and then ADP (state 3, complex I) are shown in Table 1. Thus, incubation of mitochondria with 20−100 μM T-4,5-D for 5 min evoked a concentration-dependent increase in the rate of state 4 oxygen consumption. However, incubation of mitochondria with higher T-4,5-D concentrations (≥200 μM) caused the rate of state 4 respiration to decrease. T-4,5-D (30−50 μM) also appeared to cause significant inhibition of state 3 oxygen consumption. However, at higher T-4,5-D concentrations, ADP addition caused no increase in the rate of oxygen consumption above that stimulated by malate with pyruvate, suggesting that complex I was completely inhibited and/or that mitochondria were fully uncoupled. The ability of T-4,5-D (≤100 μM) to stimulate state 4 and inhibit state 3 oxygen consumption resulted in a concentration-dependent decrease of the respiratory control ratio (RCR) that reached unity at ≥100 μM. Addition of succinate restored oxygen consumption (complex II respiration) when mitochondria were incubated for 5 min with ≤50 μM T-4,5-D. However, at higher T-4,5-D concentrations, succinate-stimulated oxygen consumption was almost completely inhibited. High concentrations of T-4,5-D (≥500 μM) profoundly inhibited state 4 and completely inhibited state 3 respiration stimulated by NAD-linked (malate with pyruvate) and FAD-linked (succinate) substrates.

Similar effects were observed when T-4,5-D was incubated with intact mitochondria for 10 min prior to addition of malate with pyruvate and then ADP. However, 10 μM T-4,5-D was sufficient to cause significant inhibition of state 3 respiration (Table 1).

Effects of T-4,5-D on NADH-CoQ_1 Reductase Activity. To confirm that T-4,5-D inhibited complex I, as suggested from oxygen electrode studies with intact

Table 2. Irreversible Inhibition of Rat Brain Mitochondrial NADH-CoQ_1 Reductase by T-4,5-D and the Influence of Antioxidants and Enzymes

T-4,5-D concentration (μM)	added antioxidant or enzyme (concentration)	NADH-CoQ_1 reductase activity[a] (% of control activity[b])
0	–	100 ± 4.1[c]
10	–	88.3 ± 1.5[d]
30	–	60.4 ± 1.8[e]
50	–	51.8 ± 2.1[e]
100	–	16.2 ± 1.0[e]
100	GSH (1 mM)	91.1 ± 1.6
100	GSH (10 mM)	97.5 ± 2.5
100	CySH (1 mM)	94.8 ± 2.8
100	CySH (10 mM)	98.6 ± 3.9
100	ascorbic acid (10 mM)	93.9 ± 3.3
100	SOD (100 units)	11.3 ± 1.2[e]
100	catalase (420 units)	31.2 ± 1.5[e]

[a] Frozen−thawed rat brain mitochondria (200 μg of protein) were incubated at 30 °C in 100 μL of 20 mM potassium phosphate buffer (pH 8) with the indicated concentrations of T-4,5-D and antioxidant or enzyme. After 60 min, 500 μL of 20 mM phosphate buffer (pH 8) was added and the resultant solution centrifuged (150000*g* for 10 min at 4 °C). The washed pellet was suspended in 100 μL of phosphate buffer and a 25 μL aliquot removed and assayed for NADH-CoQ_1 reductase activity. [b] Data are means ± SEM ($n \geq 5$). [c] Control acitivity of 291.7 ± 14.0 nmol of NADH min^{-1} mg of mt $protein^{-1}$. [d] $p < 0.05$. [e] $p < 0.005$.

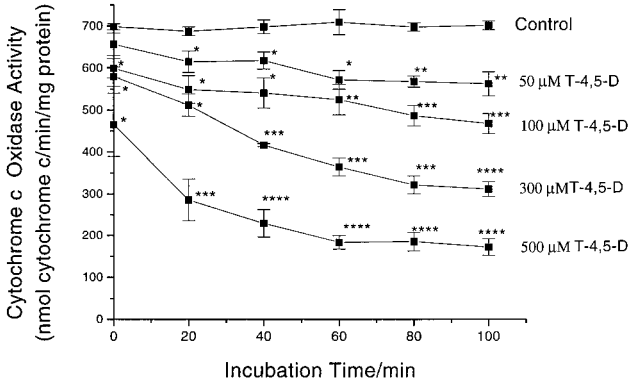


Figure 2. Time-dependent effects of T-4,5-D on cytochrome *c* oxidase activity. Rat brain mitochondrial membranes (2.5 μg of protein/μL) were incubated in 20 mM potassium phosphate buffer (pH 7.4) in the absence (control) and presence of T-4,5-D at the specified concentration. Aliquots were assayed at various times for COX activity as described in Method III in Materials and Methods. Data are means ± SEM (vertical bars) ($n \geq 3$). *$p < 0.05$. **$p < 0.005$. ***$p < 0.0005$. ****$p < 0.0001$.

mitochondria (Table 1), its effects on NADH-CoQ_1 reductase activity were studied using frozen−thawed rat brain mitochondria (mt membranes). T-4,5-D evoked a time- and concentration-dependent inhibition of rotenone-sensitive NADH-CoQ_1 reductase activity (Figure 1). Using assay method I, T-4,5-D at concentrations as low as 30 μM caused statistically significant inhibition of NADH-CoQ_1 reductase after incubation for ≥20 min with mt membranes. This inhibition developed more rapidly with higher T-4,5-D concentrations.

When mt membranes were washed, following incubation for 60 min with various concentrations of T-4,5-D (≥10 μM), NADH-CoQ_1 reductase activity remained significantly inhibited (Table 2). These results indicate that T-4,5-D evokes irreversible inhibition of NADH-CoQ_1 reductase. Large molar excesses of GSH, CySH, and ascorbic acid completely blocked the irreversible inhibition of NADH-CoQ_1 reductase by T-4,5-D. However, SOD or catalase failed to provide significant protection against

Table 3. Irreversible Inhibition of Rat Brain Mitochondrial Cytochrome *c* Oxidase by T-4,5-D and the Influence of Antioxidants and Enzymes

T-4,5-D concentration (μM)	added antioxidant or enzyme (concentration)	cytochrome *c* oxidase activity[a] (% of control activity[b])
0	–	100 ± 2.2[c]
50	–	87.9 ± 2.0[d]
100	–	62.3 ± 6.3[e]
300	–	47.8 ± 3.3[f]
500	–	24.6 ± 1.7[f]
300	GSH (3 mM)	91.2 ± 4.8
300	CySH (3 mM)	98.7 ± 1.7
300	SOD (100 units)	67.2 ± 1.3[f]
300	catalase (420 units)	58.4 ± 2.5[f]

[a] Frozen−thawed rat brain mitochondria (200 μg of protein) were incubated at 30 °C in 250 μL of 20 mM potassium phosphate buffer (pH 7.4) with the indicated concentrations of T-4,5-D, antioxidant, or enzyme. After 60 min, 400 μL of phosphate buffer (pH 7.4) was added and the resultant solution centrifuged (150000*g* for 4 °C at 10 min). The washed pellet was suspended in 500 μL of phosphate buffer and a 20 μL aliquot removed and assayed for COX activity. [b] Data are means ± SEM ($n \geq 3$). [c] Control activity of 693.3 ± 15.7 nmol of ferrocytochrome *c* min^{-1} mg of mt $protein^{-1}$. [d] $p < 0.05$. [e] $p < 0.005$. [f] $p < 0.0005$. [g] $p < 0.0001$.

Table 4. Effects of Ascorbic Acid on the Irreversible Inhibition of Rat Brain Mitochondrial Cytochrome *c* Oxidase by T-4,5-D

T-4,5-D concentration (μM)	ascorbic acid concentration (mM)	added antioxidant or enzyme (concentration)	cytochrome *c* oxidase activity[a] (% of control activity[b])
0	0	–	100 ± 5.6[c]
50	0	–	87.8 ± 2.1[d]
50	3	–	50.7 ± 1.5[f]
50	10	–	35.7 ± 1.3[g]
100	3	–	30.7 ± 1.7[g]
100	10	–	10.5 ± 1.2[g]
100	3	SOD (100 units)	93.3 ± 2.8
100	3	catalase (420 units)	77.6 ± 6.9
100	3	catalase (2000 units)	81.7 ± 1.8[d]
100	3	CySH (3 mM)	82.6 ± 2.7[d]
100	10	CySH (3 mM)	61.2 ± 4.4[d]
100	3	GSH (3 mM)	80.4 ± 2.3[d]
100	10	GSH (3 mM)	83.2 ± 1.1[d]
100	3	mannitol (5 mM)	48.1 ± 3.8[d]

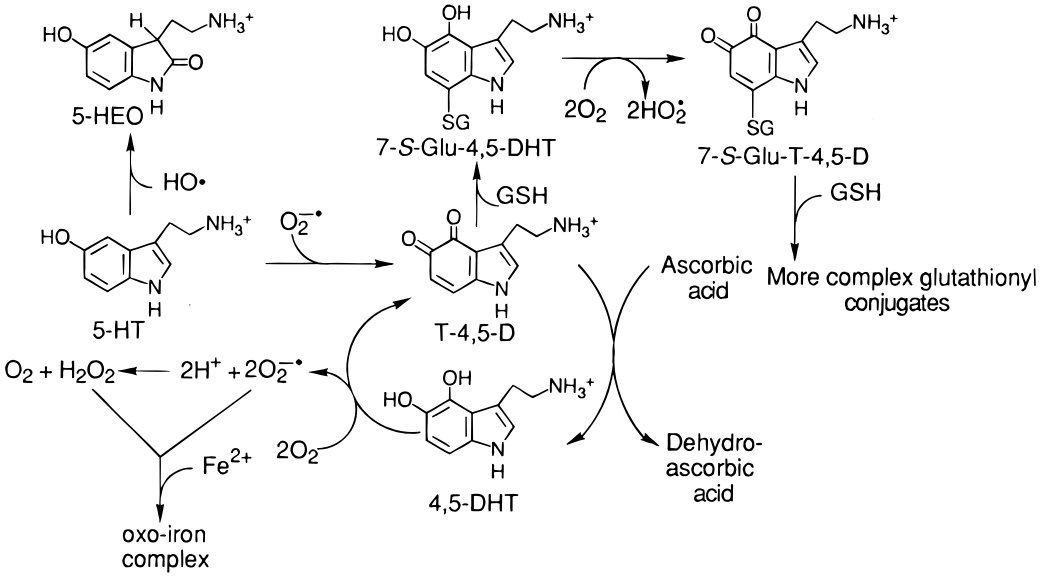
[a] See Table 3 for the procedure that was employed. [b] Data are means ± SEM ($n \geq 3$). [c] Control activity of 698.4 ± 7.1 nmol of ferrocytochrome *c* min^{-1} mg of mt $protein^{-1}$. [d] $p < 0.05$. [e] $p < 0.005$. [f] $p < 0.0005$. [g] $p < 0.0001$.

the irreversible inhibition of NADH-CoQ_1 reductase by T-4,5-D.

Effects of T-4,5-D on COX Activity. When incubated with rat brain mt membranes, T-4,5-D evoked a time- and concentration-dependent inhibition of COX (Figure 2). When the mt membranes were washed following incubation for 60 min with T-4,5-D (≥50 μM), COX remained significantly inhibited (Table 3). Excess GSH or CySH blocked this irreversible inhibition of COX by T-4,5-D. However, SOD (≥100 units) or catalase (≥420 units) was unable to protect COX against irreversible inhibition by T-4,5-D (Table 3).

Ascorbic acid potentiated the irreversible inhibition of COX by T-4,5-D in a concentration-dependent fashion that could be blocked by SOD (≥100 units) and significantly attenuated by catalase (Table 4). Excess GSH or CySH also attenuated the irreversible inhibition of COX by T-4,5-D in the presence of ascorbic acid, although they were unable to completely block this inhibition. The hydroxyl radical ($HO^{\bullet}$) scavenger mannitol (≥5 mM) provided little protection against the irreversible inhibi-

Scheme 1



tion of COX when mt membranes were incubated for 60 min in the presence of T-4,5-D and ascorbic acid (Table 4).

Discussion

Previous studies have demonstrated that T-4,5-D reacts rapidly with the sulfhydryl (SH) residue of GSH to give, initially, 7-*S*-glutathionyl-4,5-dihydroxytryptamine (7-*S*-Glu-4,5-DHT) which is readily autoxidized to 7-*S*-glutathionyltryptamine-4,5-dione(7-*S*-Glu-T-4,5-D)(Scheme 1) (*47*). 7-*S*-Glu-T-4,5-D can react further with GSH to give more complex glutathionyl conjugates (*47*). T-4,5-D also binds to SH residues of alcohol dehydrogenase (*44*) and guanine nucleotide-binding regulatory proteins (*50*) with resultant inhibition of their activities. Modifications of SH (cysteinyl) residues of mt respiratory enzyme complexes can evoke uncoupling (*51*, *52*) and loss of function (*53*). Thus, it is likely that covalent attachment of T-4,5-D to certain SH residues of mt respiratory enzyme complexes is responsible for uncoupling of respiration (Table 1) and irreversible inhibition of complex I (Table 2) and complex IV (Table 3).

The inability of SOD or catalase to attenuate the irreversible inhibition of NADH-CoQ_1 reductase (Table 2) or COX (Table 3) by T-4,5-D indicates that damage to these respiratory enzyme complexes is probably not mediated by $O_2^{-\bullet}$, H_2O_2, or $HO^\bullet$. The protection afforded by GSH and CySH reflects the ability of these compounds to scavenge T-4,5-D (*47*) and prevent covalent modification of key SH residues at or close to the complex I (*54*) and complex IV (*55*) sites. Indeed, 7-*S*-Glu-T-4,5-D (300 μM) was unable to evoke irreversible inhibition of COX when incubated for 60 min with mt membranes.

Ascorbic acid potentiates the irreversible inhibition of COX by T-4,5-D (Table 4). However, SOD protects COX against irreversible inhibition by T-4,5-D in the presence of ascorbate, and catalase significantly attenuates this inhibition. Thus, it can be concluded that the mechanisms underlying the irreversible inhibition of COX by T-4,5-D in the presence and absence of ascorbate are different. On the basis of earlier studies (*49*), it is probable that ascorbic acid reduces T-4,5-D to 4,5-dihydroxytryptamine (4,5-DHT) that is then oxidized back to T-4,5-D by molecular oxygen, thus establishing a redox cycling system that generates $O_2^{-\bullet}$ and then H_2O_2 (Scheme 1). Decomposition of H_2O_2 by trace concentrations of Fe^{2+} or certain other transition metal ions and generation of $HO^\bullet$ (*7*) might be responsible for irreversible damage to COX. However, the observation that mannitol does not protect COX against irreversible inhibition by T-4,5-D in the presence of ascorbic acid (Table 4) tends not to support a role for $HO^\bullet$. Furthermore, SOD, which catalyzes decomposition of $O_2^{-\bullet}$ to H_2O_2, the essential precursor of $HO^\bullet$ by Fenton/Haber-Weiss chemistry (*7*), blocks the irreversible inhibition of COX by T-4,5-D in the presence of ascorbate (Table 4). These results suggest that $HO^\bullet$ is probably not the major species responsible for irreversible damage to COX. However, catalase also significantly attenuates the irreversible inhibition of COX by T-4,5-D in the presence of ascorbate (Table 4). Thus, cogeneration of $O_2^{-\bullet}$ and H_2O_2 by the ascorbate-mediated redox cycling of T-4,5-D appears to be necessary to evoke irreversible inhibition of COX. This raises the possibility that a redox cycling oxo−iron species, formed by reaction of trace levels of iron with cogenerated $O_2^{-\bullet}$ and H_2O_2 (*56*), is responsible for damage to COX. Such a putative oxo−iron species appears to selectively damage COX because excess ascorbic acid completely protects NADH-CoQ_1 reductase against irreversible damage by T-4,5-D (Table 2). It therefore appears likely that the protective effects of ascorbic acid against inhibition of NADH-CoQ_1 reductase reflect its ability to maintain T-4,5-D predominantly as 4,5-DHT and hence block its covalent modification of key SH residues at the complex I site.

The release and subsequent reuptake of 5-HT evoked by MA (*15*) and I-R (*19*), elevation of extracellular levels of Glu (*17*, *20*, *21*) with resultant NMDA receptor activation, particularly under conditions of reduced neuronal energy metabolism (*12*, *13*, *18*), and intraneuronal $O_2^{-\bullet}$ generation (*23*, *24*) together provide conditions for oxidation of elevated cytoplasmic concentrations of 5-HT to T-4,5-D. However, $O_2^{-\bullet}$ spontaneously dismutes to H_2O_2, the essential precursor of $HO^\bullet$ (*7*), raising the possibility that the latter radical might initiate the neurodegenerative processes evoked by MA and I-R. Several lines of evidence argue against this possibility.

For example, Cu/Zn SOD transgenic mice are resistant to MA (*25*, *26*)- and reperfusion-induced (*27*) neuronal injury. This implies an essential role for $O_2^{-\bullet}$ but not H_2O_2 and thence $HO^\bullet$ in the neurodegenerative mechanisms. Furthermore, the $HO^\bullet$-mediated oxidation of 5-HT generates 5-hydroxy-3-(ethylamino)-2-oxindole (5-HEO) as the major and most stable product (Scheme 1) (*57*). However, 5-HEO levels in rat brain were not increased 1 h after administration of a large neurotoxic dose of MA (*58*), a result that argues against the participation of $HO^\bullet$ at least in the early stages of the neurotoxic processes evoked by MA. Nevertheless, 1 h after such a large dose of MA, levels of 5-HT were dramatically reduced, particularly in the hippocampus and cortex (*58*). These 5-HT level decreases may, in part at least, reflect the inhibition of tryptophan hydroxylase by MA (*59*). However, the loss of 5-HT was not accompanied by corresponding increases in the levels of its metabolite 5-hydroxyindole-3-acetic acid, pointing perhaps to other mechanisms that might account for depletion of the neurotransmitter such as its oxidation by $O_2^{-\bullet}$ to T-4,5-D. It remains to be experimentally established that MA or I-R does in fact mediate the latter reaction.

The results of this investigation suggest that in the absence of ascorbic acid intraneuronal T-4,5-D could, depending on concentration, uncouple mt respiration or inhibit complexes I, II, and IV. However, a recent study has demonstrated that neurons normally contain approximately 10 mM ascorbic acid (*60*). Such a high concentration of ascorbic acid would be expected to protect NADH-CoQ_1 reductase, and hence complex I respiration, against irreversible inhibition by T-4,5-D (Table 2). Furthermore, by maintaining T-4,5-D largely as 4,5-DHT, ascorbic acid would also be expected to protect SH residues of other mt respiratory enzyme complexes against covalent modification. However, ascorbic acid potentiates the irreversible inhibition of COX by T-4,5-D (Table 4). Thus, intraneuronal $O_2^{-\bullet}$, formed as a result of MA administration or I-R, might initially oxidize cytoplasmic 5-HT to T-4,5-D that undergoes ascorbate-dependent redox cycling reactions cogenerating $O_2^{-\bullet}$ and H_2O_2. The results presented here cannot entirely discount direct roles for $O_2^{-\bullet}$, H_2O_2, and thence $HO^\bullet$ in evoking irreversible inhibition of COX. However, a species that is more likely responsible for COX inhibition is a redox cycling oxo−iron complex (*56*). Such a sequence of reactions might contribute to the inhibition of COX evoked by MA (*43*) and I-R (*41*) with the resultant decreased level of ATP production and the degeneration of serotonergic neurons.

The neuronal damage evoked by MA affects not only serotonergic and dopaminergic neurons but also Glu cells in the somatosensory cortex (*61*), a brain region innervated by 5-HT terminals (*62*). I-R also leads to more widespread neurodegeneration, particularly to CA1 cells in the hippocampus. Furthermore, ischemia-induced release of 5-HT clearly plays a role in CA1 neuronal cell death (*19*). It is relevant, therefore, that a number of neuronal pre- and postsynaptic elements have high-affinity 5-HT uptake systems (*63*). This raises the possibility that uptake of 5-HT by nonserotonergic neurons and NMDA receptor activation by elevated extracellular levels of Glu might mediate intraneuronal oxidation of 5-HT by $O_2^{-\bullet}$ to T-4,5-D that contributes to the inhibition of mt respiratory enzyme complexes and degeneration of such cells.

In summary, T-4,5-D, the major product of the in vitro oxidation of 5-HT by $O_2^{-\bullet}$, is a mt toxicant that in the absence of ascorbic acid irreversibly inhibits NADH-CoQ_1 reductase and COX, probably by covalent modification of key SH residues of these respiratory enzyme complexes. In the presence of excess ascorbic acid, T-4,5-D may redox cycle in a reaction that potentiates formation of $O_2^{-\bullet}$ and H_2O_2 and thence an ill-defined oxo−iron complex that selectively and irreversibly damages COX. Because of the apparent involvement of 5-HT, $O_2^{-\bullet}$, and inhibition of COX in the degeneration of serotonergic and perhaps other neurons evoked by MA and I-R, it is possible that T-4,5-D might be an aberrant metabolite that contributes to the underlying neurodegenerative mechanisms.

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