Differential Mechanisms in the Effects of Disulfiram and Diethyldithiocarbamate Intoxication on Striatal Release and Vesicular Transport of Glutamate

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ABSTRACT

Intoxication with the alcohol-aversive drug disulfiram (Antabuse) and related dithiocarbamates may provoke neuropathies and, in some cases, damage the basal ganglia. Rats received a single administration of disulfiram (7 and 500 mg kg⁻¹ i.p.) and equimolar doses (4 and 290 mg kg⁻¹ i.p.) of its metabolite diethyldithiocarbamate (DDC), roughly corresponding to the daily maximum dose in alcohol abusers or to an estimated nonlethal overdose, respectively. The striatal, extra-cellular levels of glutamate in freely moving rats previously implanted with a microdialysis probe increased after low and intoxicating doses of disulfiram (126 ± 3% and 154 ± 10% of basal values, respectively) and DDC as well (135 ± 10% and 215 ± 14%, respectively), a partially Ca⁺⁺-dependent effect. The prolonged (>7 hr) disulfiram-induced increase in glutamate observed in vivo may reflect the in vitro disulfiram-evoked release of glutamate from striato-cortical synaptic vesicles, where the drug nonspecifically inhibited (Kᵢ = 4 μM) the uptake function and abolished the transmembrane proton gradient (ΔpH). In contrast, DDC did not seem to affect ΔpH. The prompt DDC-provoked increase in extracellular levels of glutamate was prevented by 7-nitroindazole, an in vivo specific inhibitor of neuronal nitric oxide synthase, which suggests that the thiol metabolite also acts via the nitric oxide synthesis. At variance, the short-acting 7-nitroindazole did not prevent the sustained in vivo effects of disulfiram and of DDC putatively formed with time. These findings provide new evidence for differential mechanisms underlying disulfiram- and DDC-induced increases in striatal glutamate release. Present glutamatergic changes, although not appearing dramatic enough to represent the only cause for neuronal damage from disulfiram overdose, might contribute to the drug neurotoxicity.

Disulfiram (Antabuse) and related dithiocarbamates are nonspecific highly reactive chemicals, because of their affinity for sulfhydryl groups and their metal-combining capacity. Disulfiram has an almost 50-year-long history of use in the aversion therapy of alcoholism, on the grounds that the unpleasant consequences of inhibiting aldehyde dehydrogenases (Veverka et al., 1997) would lead to a lasting distaste for alcohol. The abnormally high circulating and tissue concentrations of acetaldehyde, disulfiram itself and major metabolites DDC and carbon disulfide are expected to provoke several side effects besides those strictly related to the aversive reaction. In fact, the inherent toxicity of disulfiram, promoted by its ability to easily enter the brain (Eneanya et al., 1981), involves several behavioral and neurological complications. The chronic treatment with disulfiram in abstinent alcoholics may provoke drowsiness, apathy, headache, psychosis, peripheral sensorimotor neuropathy and optic neuritis, whereas encephalopathy, cerebral seizures and extrapyramidal syndromes have been observed more frequently in patients with nonlethal overdoses (see Ellenhorn et al., 1997). Lesions of the basal ganglia underlying the extrapyramidal symptoms caused by disulfiram intoxication, and rarely, by long-term therapy have been described (Lidy et al., 1979; Hirschberg et al., 1987; Krauss et al., 1991; Laplane et al., 1992; Riley, 1992; De Mari et al., 1993). This led to the hypotheses for a copper-dependent oxidative stress caused by abnormal metal accumulation in selected brain regions of disulfiram-treated rats (Delmaestro, 1995), and/or for the induction of necrotic/apoptotic cell death provoked by expo-

ABBREVIATIONS: DDC, diethyldithiocarbamic acid; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid; NOS, brain nitric oxide synthase; 7-NI, 7-nitroindazole; ΔpH, transmembrane electrochemical-proton gradient; Δφ, transmembrane potential gradient; ΔH⁺, transmembrane proton gradient; DMSO, dimethyl sulfoxide; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; NMDA, N-methyl-D-aspartate; ANOVA, analysis of variance.
sured to redox-active agents such as dithiocarbamate toxicants and disulfiram-like thiuram disulfides (Orrenius et al., 1996). The suggested disulfiram-induced impairment of catecholaminergic transmission (Fisher, 1989; De Mari et al., 1993; Zorzon et al., 1995) in the origin for neurologic complications seems to have been overlooked (Vaccari et al., 1996). Because dithiocarbamates are highly nonspecific in action, it is difficult to identify a single mechanism underlying their neurotoxic effects.

In the present in vivo and in vitro study, we provide new evidence that both intoxicating and low, single doses of disulfiram and DDC increase striatal extracellular levels of glutamate, an excitatory neurotransmitter which has potentially neurotoxic effects when released in large excess of physiological concentrations (Choi, 1988; Lipton and Rosenberg, 1994; for a review, see Ohrenvitch and Urenjak, 1997).

Materials and Methods

Materials. Male Sprague-Dawley rats (300–350 g) were used for in vivo and in vitro experiments. They were housed under a 12-hr light/dark cycle (lights on at 6 P.M.) and in a temperature- and humidity-controlled environment with free access to water and food. l-2,3-3HGlutamic acid (specific activity, ~49 Ci mmol−1) and [14C]methyamine (specific activity, 54 mCi mmol−1) were purchased from Amersham Corp. (Little Chalfont, UK). Tetraethylthiuram disulfide (disulfiram), DDC sodium salt, 7-NI, FCCP and all other compounds were obtained from Sigma Chemical Co., St. Louis, MO, with the exception of PDC which was purchased from RBI-Amersham (Little Chalfont, UK).

Microdialysis procedure. The animals, anesthetized with a 1.5% mixture of halothane and air, were mounted in a David Kopf stereotaxic frame with the upper incisor bar set at −2.5 mm below the intra-aural line. A microdialysis probe of concentric design (0.5 mm outer diameter, 4 mm length; CMA 12 Carnegie Medicin AB, Stockholm, Sweden) was implanted stereotaxically into the right or left neostriatum (coordinates: A: +0.3; L: ±3.1; V: −8.5, from the bregma and the dura surface, respectively) (Paxinos and Watson, 1982). Thereafter, the probe was secured permanently to the skull with stainless steel screws and methacrylic cement, and the animals were allowed to recover for 36 hr before starting the experiments. On the day of the release assays, the microdialysis probe was perfused at a flow rate of 2 μl min−1 with Ringer’s solution (in mM: Na+, 147; K+, 4; Ca2+, 2.4; Cl−, 156; glucose, 2.7), and after a 5-hr period for stabilization of the base line, perfusates were collected every 20 min. After three stable basal values had been obtained, disulfiram or DDC were administered.

Glutamate analysis. For glutamate measurements, 10–μl aliquots of each perfusate sample were used. The amino acid assay was based on precolumn derivatization with an o-phthalaldehyde/dehydromercaptoethanol reagent and separation by reverse-phase high-performance liquid chromatography on a 5-μm Nucleosil 100 (C18) column, followed by fluorometric detection (wavelengths: emission, 450 nm; excitation, 370 nm). The mobile phase consisted of 0.1 M sodium acetate, 0.1 mM ethylenediaminetetraacetic acid, 8% methanol adjusted to pH 3.8 and 1.5% tetrahydrofuran. The flow rate was 1 ml min−1. The limit of detection was 5 nmol/sample (Morari et al., 1993).

Drug administration. Disulfiram and DDC were freshly dissolved in a 1:1 (v/v) propylene glycol/DMSO solution (vehicle). Freely moving rats with a microdialysis probe implanted in the striatum received disulfiram either at a low therapeutic-like or at a high intoxicating dose (7 and 500 mg kg−1 i.p., respectively). They corresponded roughly to a maximum maintenance dose of 500 mg day−1 in alcohols (Brewer, 1993; Ellenhorn et al., 1997), or to the estimated amount of drug taken in four cases of voluntary, nonlethal intoxication (Hirschberg et al., 1987; Laplane et al., 1992; Del Maestro, 1995; Zorzon et al., 1995). Equimolar doses (4 and 290 mg kg−1 i.p., respectively) of the thiol metabolite DDC also were administered. When required, the animals were injected with 30 mg kg−1 i.p. of 7-NI, 20 min before the drugs according to its relatively short-lived inhibition of brain NOS activity (Bahbudge et al., 1993; Salter et al., 1996). When administered alone, this dose had no effect on the spontaneous electrophysiological activity in rat striatum in vivo (Schultz et al., 1995).

Preparation of brain synaptic vesicles. Synaptic vesicles obtained from pooled tissues (entire cortex and/or striatum, at least 1 g of tissue) were prepared according to a simplified procedure (Kish and Ueda, 1989) involving 1:10 (v/v) homogenization in a solution containing 0.32 M sucrose, 0.5 mM calcium acetate, 1 mM magnesium acetate, 1 mM NaHCO3, with a Teflon-glass homogenizer. The homogenates were centrifuged for 15 min at 12,000 × g (4°C, Sorvall SS-34 rotor). The resulting pellets were resuspended gently in 10 vol of ice-cold lysing solution (6 mM Tris-maleate, pH 8.1) for 45 min, and then centrifuged for 15 min at 43,000 × g. Supernatants then were spun for 55 min at 200,000 × g (Beckman 50 Ti rotor). The final pellets were resuspended in 0.32 M sucrose, 1 mM NaHCO3, and 1 mM dithiothreitol solution. The crude synaptic vesicles were stored at −70°C and used within 2 weeks from their preparation. During this time no appreciable loss of glutamate uptake activity occurred.

Assay of vesicular, ATDP-dependent uptake and release of [3H]glutamate. For the uptake of glutamate (Kish and Ueda, 1989), duplicate aliquots (40–50 μg) of cortico and striatal vesicular proteins were preincubated in 80 μl of medium (0.25 M sucrose, 4 mM MgSO4, 5 mM Tris-maleate, pH 7.4, 4 mM KCl, 2 mM potassium aspartate) for 5 min at 30°C in the absence or presence of freshly DMSO-dissolved test compounds. Control samples contained an equal volume (2 μl) of DMSO. After preincubation the uptake was initiated by the addition of a mixture (final concentration, 50 μM) of unlabeled and [3H]glutamate, and 2 mM ATP (neutralized with Tris base). After incubation at 30°C for 10 min the uptake was stopped by the addition of 2 ml of ice-cold 0.15 M KCl and immediate filtration through glass-fiber GF/F filters (previously soaked for 1 hr in a 1% polyethyleneimine solution). Test tubes were rinsed with 2 ml of KCl solution three more times, and the filters were washed an additional four times with the same solution. The values of [3H]glutamate uptake obtained from vesicles incubated over ice (blanks) were subtracted from corresponding samples at 30°C.

For efflux experiments, preincubated pools of cortico-striatal vesicles were replenished with 50 μM unlabeled plus [3H]glutamate and ATP for 5 min, a time when equimolar concentrations of test compounds were added to the vesicle suspension. Thereafter, samples were filtered at 10, 30 or 40 min of incubation.

Vesicular uptake of [14C]methyamine. The ATP-dependent uptake of 50 μM [14C]methyamine into cortico-striatal vesicles was measured as a putative index of the transmembrane proton gradient (∆pH) which is proportional to the accumulation (Tabb et al., 1992). The assay medium contained 0.14 M potassium gluconate instead of sucrose, plus 20 mM HEPES (pH 7.4), 4 mM MgSO4, 80 to 100 μg of vesicle proteins and 2 mM Tris-ATP. Vesicles were preincubated for 1 hr at 4°C in the above-mentioned buffer, after which the uptake of 50 μM [14C]methyamine was run for 5 min at 30°C.

Statistics. Response data (means ± S.E.) for in vivo experiments were reported as percent changes from base line (mean of three samples collected before treatments). The significance of differences regarding the peak effects (maximal responses) was indicated. In
addition, the area under the time-response curve representing the integrated response over time was calculated for each animal. The area values (overall effects) were expressed as percentage changes in arbitrary units. The statistical analysis was carried out with one-way ANOVA followed by post hoc tests for multiple comparisons. In table 1, in which two groups of data were compared, the Student’s t test was used.

Results

In vivo release of glutamate. Basal glutamate release from the striatum was 0.4 ± 0.05 μM (n = 64). Because the absolute values in the saline and propylene glycol/DMSO vehicle-treated animals were similar and remained stable throughout the release experiment, they were pooled together (n = 22) for statistical comparisons. The administration of disulfiram (500 mg kg⁻¹) was associated with a prompt (maximum peak, 154 ± 10% of basal values, n = 9) and prolonged increase in glutamate release (fig. 1a). The facilitatory effect of disulfiram was still present 2 hr (fig. 1a) and 7 hr after the injection of the drug (134 ± 5%, n = 4, data not shown). The 7 mg kg⁻¹ dose of disulfiram induced a slight increase in the amino acid release, which was significant when analyzing the maximal peak effect (126 ± 3%, n = 8) as well as the area-under-the-curve values, which mainly reflects the overall effect of the drug (fig. 1a, right panel). The administration of DDC (290 mg kg⁻¹) increased glutamate release with a maximal effect (215 ± 14%, n = 7) 20 min after the injection; thereafter, the effect declined rapidly (fig. 1b). When DDC was administered at the 4 mg kg⁻¹ dose, the increase in glutamate release was less pronounced but still significant in respect to the peak effect (135 ± 10%, n = 7) and the overall effect (fig. 1b, right panel). The pretreatment (20 min) with 7-NI (30 mg kg⁻¹ i.p.), an in vivo selective inhibitor of the neuronal isoform of NOS (Moore et al., 1993a; Babbedge et al., 1993), which when administered alone was ineffective on the striatal glutamate release (n = 8), fully counteracted the DDC (290 mg kg⁻¹, n = 6)-evoked release (fig. 2b), but not the disulfiram (500 mg kg⁻¹, n = 6)-induced increase in striatal glutamate release (fig. 2a).

The enhancement of glutamate release induced by the higher doses of disulfiram and DDC (500 mg kg⁻¹ and 290 mg kg⁻¹, respectively) was reduced partially during the local perfusion with a Ca⁺⁺-free Ringer’s solution (table 1).

In vitro effects on the vesicular uptake and release of glutamate. Disulfiram potently inhibited [³H]glutamate uptake in striatal and cortical synaptic vesicles, with similar affinity values in the low micromolar range (table 2). DDC and carbon disulfide, the potent inhibitor of the synaptic plasma membrane transporter for glutamate PDC, and the less potent and nonselective dihydrokainic acid (Johnston et al., 1979; Bridges et al., 1991) were poorly active, with Ki values exceeding 1 mM (table 2). Disulfiram inhibition was noncompetitive, as indicated by decreased Vmax and similar Km values (fig. 3). To further characterize the mechanism by which test compounds could release endogenous glutamate, isolated vesicles previously replenished with 50 μM glutamate were incubated in the absence or presence of equimolar concentrations of thiols, which matched the in vivo therapeutic and intoxicating dose range on assuming their uniform distribution in the body. No appreciable spontaneous leakage of glutamate from the vesicles was observed after 10 to 40 min of incubation, when the radioactivity left in vesicles had reached a maximum plateau (fig. 4). The lower (24 μM) concentration of DDC did not affect the vesicle [³H]glutamate content, whereas disulfiram decreased it to 46 ± 4% of controls. Disulfiram and DDC (1.7 mM) lowered the vesicle radioactivity at 40 min of incubation to 32 ± 3% and 53 ± 8% of controls, respectively (fig. 4).

![Fig. 1. Effects of disulfiram (a) and DDC (b) on striatal glutamate release in awake rats.](image)
Vesicular uptake of $[14C]$methylamine. The ATP-dependent $[14C]$methylamine uptake in cortico-striatal vesicles ($n = 5$) was abolished by the higher concentration of disulfiram, whereas DDC was inactive (fig. 5a). Incubation of $[3H]$glutamate-replenished vesicles with FCCP, a dissipater of the proton-electrochemical gradient ($\Delta f = \Delta \psi - \Delta \phi$), was equipotent with disulfiram in decreasing (to $42 \pm 1.7\%$ of controls) the vesicular radioactivity, whereas DDC only decreased it to $74 \pm 1.2\%$ of controls (fig. 5b). The addition of disulfiram stimulated modestly (by $13\%$), although significantly ($P < .01$), the FCCP-induced loss of glutamate, the likely result of disulfiram-promoted additional dissipation of $\Delta f$. Most of (FCCP + DDC)-provoked loss of glutamate seemed to depend on FCCP; the small DDC-related component could represent either further dissipation of $\Delta \phi$ or other unidentified causes.

**Discussion**

The present results show that both a low dose and a non-lethal overdose of disulfiram and DDC, its major metabolite, increase striatal glutamate release in awake, freely moving rats. This brain region also receives a major glutamatergic input from the neocortex and subthalamic nucleus and is densely populated by NMDA subtype and additional receptors for excitatory amino acids (Albin et al., 1992).

Although it is usually difficult to discriminate between neuronal vs. glial and/or metabolic glutamate in microdialysis experiments (Westerink, 1995; Herrera-Marschitz et al.,...
the demonstration that the effects of disulfiram and DDC were partially decreased during the local perfusion with disulfiram, but not DDC. Because DDC does not affect the alteration of the redox state of membranes of bovine adrenal chromaffin granules (Schlichter et al., 1975). Similar states of energy deprivation have been linked to neuronal excitotoxic damage (Greene and Greenamyre, 1996).

The different time course of DDC vs. disulfiram-induced glutamate release, and the finding that in vivo neuron-specific (Moore et al., 1993a; Babbedge et al., 1993) NOS inhibition by 7-NI almost totally prevented the effects of DDC while being ineffective against disulfiram, suggest that the two compounds might operate via different mechanisms as well as extravesicular and extraneuronal pools (Herrera-Marschitz et al., 1996). In this context, DCC appeared to release glutamate mainly via NO synthesis, in the absence of an effect on vesicular uptake. This finding supports the idea that NO is a local signal facilitating the presynaptic release of glutamate which, in turn, activates striatal NO production (Garthwaite, 1991; Guevara-Guzman et al., 1994; Bogdanov and Wurtman, 1997). However, the inability of 7-NI to attenuate disulfiram effects, despite the increasing presence of the reduction product DDC and the drug nonspecific glutamate-activated synthesis of NO, suggest that the amounts of endogenous DDC produced shortly after disulfiram injection probably were not sufficient to influence local glutamate increases significantly. Thus, although DCC may be involved in the long lasting effects of disulfiram, significant NO production may coincide only with periods when the short-lived NOS inhibition already had faded. In fact, maximal inhibition of cerebellar and striatal NO activity occurs within 30 min after the i.p. injection of 7-NI, the enzyme activity then approaching normality 2 hr later (Moore et al., 1993b; Kalisch et al., 1996).

Disulfiram and, to a lesser extent DDC, partially depleted [H]glutamate from isolated cortico-striatal vesicles. The ATP-dependent [14C]methylamine uptake in cortico-striatal vesicles, a putative index (Tabb et al., 1992) for the transmembrane proton (ΔpH) gradient needed to drive glutamate uptake, was concentration-dependently inhibited by disulfiram and DDC. Because DDC does not affect ΔpH (a), its activity on Δψ and/or additional mechanisms might be inferred. FCCP was added at 5 min of incubation to duplicate aliquots (n = 3 individual experiments) of cortico-striatal vesicles replenished with [3H]glutamate plus ATP. Test compounds, when requested, were added at 10 min and the incubation was stopped at 30 min. Newman-Keuls test for multiple comparisons after one-way ANOVA indicated that all treatments differed (**P < .01) from controls. Furthermore, (FCCP + DDC) differed from FCCP (P < .01), and (FCCP + disulfiram) from both FCCP and disulfiram (P < .01).

The prolonged (>7 hr) disulfiram-induced increase in glutamate release was consistent (see Velasco et al., 1996) with the progressive accumulation of extracellular glutamate, which is hindered from re-entering the synaptic vesicles, as observed in vitro. In this respect, it seems relevant that poorly selective disulfiram, at variance with DDC, also found to antagonize synaptosomal dopamine and glutamate uptake nonspecifically via the alteration of the redox state of membrane thiols (Di Monte et al., 1989), and the inhibition of both (Na⁺,K⁺)- and vesicle-related (Mg⁺⁺)-ATPases (Mamatha and Nagendra, 1994), respectively. Energy impairment also was found with disulfiram (but not DDC)-provoked inhibition of Mg⁺⁺/ATP-dependent uptake of dopamine in membranes of bovine adrenal chromaffin granules (Schlichter et al., 1975). Similar states of energy deprivation have been linked to neuronal excitotoxic damage (Greene and Greenamyre, 1996).
Contribute, as a whole, to the clinical neurotoxicity of disulfiram. Considering the recovery factor (12 ± 1.3%) for glutamate across the dialysis membrane, basal extracellular levels of the amino acid were brought by higher doses of disulfiram and DDC from approximately 3 μM up to top values of 5 and 7 μM, respectively. This represents only a small fraction of the estimated total vesicular content (~100 mM) of glutamate (Nicholls, 1993). Unlike in vitro models, in which the neurotoxic threshold for glutamate is set at approximately 1 to 2 μM (Meldrum and Garthwaite, 1990), it is not clear how much extracellular glutamate is necessary in vivo to damage the neurons. Recently, 8 μM glutamate was measured in the cerebrospinal fluid of patients with progressive ischemic stroke (Castillo et al., 1997). However, the present glutamate changes were approximately 20- to 50-fold lower than those reported as occurring in most experimental studies where neurotoxicity was implied (see Obrenovitch and Urenjak, 1997). It has been calculated that during experimental ischemia and anoxia, extracellular glutamate levels equilibrate at 370 μM, a concentration “high enough” to kill neurons (Bouvier et al., 1992). Therefore, taking for granted also that “high extracellular levels of glutamate do not necessarily produce neuronal dysfunction and death in vivo…” (Obrenovitch and Urenjak, 1997), the present changes in glutamate would not be conclusive evidence for a pathogenic role of disulfiram. Nevertheless, disulfiram overdosage is more likely to occur in human alcoholics, where the glutamatergic neurotransmission purportedly is impaired, NMDA receptors are supersensitive (Tsai et al., 1995) and the compound aggravates a preexistent state of energy deprivation. All these conditions (Greene and Greenamyre, 1996), as well as the drug-induced (Di Monte et al., 1989) and idiopathic failures of neuroprotective glial and neuronal reuptake functions (Rothstein et al., 1993; Rothstein, 1996; Marsilia et al., 1996), and neuronal excess of NO (Lustig et al., 1992; Dawson et al., 1993), also contribute to markedly enhanced glutamate neurotoxicity. Furthermore, the persistence of lipophilic disulfiram in body tissues also is associated with the production of another potently neurotoxic metabolite, carbon disulfide (Kane, 1970; Rainey, 1977; Eneanya et al., 1981; Huang et al., 1996). Last but not least, we administered to rats, on the basis of body weight, “human-equivalent” doses which, however, were approximately 6.3-fold smaller than in humans when expressed (Chodera and Feller, 1978; Eaton and Klaassen, 1996) in body surface area-to-weight ratios to account better for interspecific pharmacokinetic differences (Chapell and Mordenti, 1991). In other words, the present in vivo results were largely underestimated.

In conclusion, the present differential influences on the vesicular transport and in vivo striatal release of glutamate are novel effects which add to the long list of recognized neurochemical properties of disulfiram and dithiocarbamate. Glutamate alterations are probably not large enough in themselves to damage the central nervous system. Nevertheless, because of the prolonged (>7 hr) increase in glutamate release, associated with the heterogeneous neurotransmitter impairment likely to be produced by nonselective thios, they might contribute to the lesions of the basal ganglia related to acute disulfiram intoxication.

References
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