Atropine Reverses Antinociception Induced by 2,5-Hexanedione in Rats

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Abstract: 2,5-Hexanedione is a n-hexane metabolite with neurotoxic properties. We have previously demonstrated that acute administration of 2,5-hexanedione causes analgesia in the tail-flick test in rats. In the present investigation, we examined the possible involvement of a cholinergic component in the 2,5-hexanedione-induced antinociception, since literature data indicate that this hexacarbon compound may act as a competitive inhibitor of acetylcholinesterase and that cholinesterase inhibitors are analgesic to rodents. Rats were treated with saline or with 5 or 25 mg/kg atropine (intraperitoneally) 10 min. before the injection of vehicle or 800 mg/kg 2,5-hexanedione (intraperitoneally). 2,5-Hexanedione caused a significant increase in tail-flick latencies at 10, 30, 60 and 90 min. after hexacarbon injection. Atropine (5 or 25 mg/kg) partially reversed the analgesia caused by 2,5-hexanedione at 60 and 90 min. When effects of 2,5-hexanedione on brain acetylcholinesterase was assessed in vitro, the results demonstrated that a competitive component is involved in enzyme inhibition. Taken together, these data support the involvement of a cholinergic (muscarinic) component in 2,5-hexanedione-induced analgesia.

Occupational or chronic environmental exposure to hexacarbon compounds induces several toxic effects both in humans and in experimental animals (Allen et al. 1975; Couri & Milks 1982; WHO 1991; Ladefoged et al. 1989; Larsen et al. 1991; Pereira et al. 1992). The most prominent effect is an axonopathy characterized by a sensory and motor impairment in the distal extremities (Abou-Donia et al. 1985; Anthony et al. 1983; Ladefoged et al. 1989; Misumi et al. 1985; Ralston et al. 1985; Rossi et al. 1982).

Although prolonged exposure to hexacarbons may induce neurotoxic effects, few data are available regarding the acute effects of these compounds. The acute treatment with 2,5-hexanedione (a n-hexane metabolite) may help find potential molecular targets that may be involved in n-hexane neurotoxicity. In vitro, 2,5-hexanedione inhibits the activity of various enzymes of the glycolytic pathway (Howland et al. 1980; Sabri et al. 1979). In contrast, these enzymes are not affected after prolonged in vivo 2,5-hexanedione administration (Pereira et al. 1991). Furthermore, 2,5-hexanedione and other hexacarbon compounds competitively inhibit the activity of purified acetylcholinesterase from Torpedo marmorata, apparently blocking the anionic site of the enzyme (Dafforn et al. 1979).

Several studies have demonstrated that cholinergic agonists as well as cholinesterase inhibitors produce antinociception in mammals (Gillberg et al. 1989; Gower 1987; Sitaram & Gillin 1977). Recently we demonstrated that acute administration of 2,5-hexanedione to adult rats causes analgesia in the tail-flick paradigm, with a dose- and time-dependent antinociceptive effect (Pereira et al. 1991).

Since cholinesterase inhibitors and cholinergic agonists as well as 2,5-hexanedione may produce antinociceptive actions (Gillberg et al. 1989; Gower 1987; Pereira et al. 1991; Sitaram & Gillin 1977) we examined the possible involvement of muscarinic receptors in 2,5-hexanedione-induced analgesia. The in vitro inhibitory effect of 2,5-hexanedione on brain acetylcholinesterase was also investigated in order to determine a possible role of the inhibition of this enzyme in the analgesia caused by 2,5-hexanedione.

Materials and Methods

Female Wistar rats from our own breeding stock (75-90 days old) weighing 170-200 g were maintained in groups of 6 in opaque plastic cages (50 cm×36 cm×18 cm). Rats had free access to water and food and were maintained on a 12 hr light/12 hr dark cycle (lights on at 7 a.m.) in an air-conditioned constant-temperature colony room. All experiments were conducted between 1 p.m. and 5 p.m.

Nociception was assessed with a tail-flick apparatus. Rats were wrapped in a towel and placed in the apparatus and the light source positioned below the tail was focused on a point about 2.0 cm rostral to the tip of the tail. Deflection of the tail automatically activated a photocell that terminated the trial. Light intensity was adjusted so that a baseline tail-flick latency of 2-4 sec. was obtained. A cut-off time of 10 sec. was used to avoid tail tissue damage.

On day 1, rats were wrapped in a towel and a baseline tail-flick latency was obtained for each subject to familiarize animals with the tail-flick and handling procedures so as to avoid any effect of novelty on nociception (Netto et al. 1987; Vendite et al. 1990). On day 2, each rat was subjected to a second baseline tail-flick latency test. After this baseline measurement, rats were injected intraperitoneally with saline or with 5 or 25 mg/kg atropine chloride. After 10 min., rats received a second injection of vehicle (120 mM NaCl containing 10 mM potassium phosphate buffer, pH 7.2) or 800 mg/kg 2,5-hexanedione (Aldrich Chemical Company, Milwaukee, WI, U.S.A.). Nociception was...
measured at 10, 30, 60 and 90 min. after 2,5-hexanedione treatment. Data were analysed by a 3 (pretreatments: 0, 5 or 25 mg/kg atro-
pine)× 2 (treatments: 0 or 800 mg/kg 2,5-hexanedione)× 5 (time sam-
ping: basal, 10, 30, 60 or 90 min.) design with the last factor treated as repeated measure. Post hoc comparisons were made when appropriate by Duncan's multiple range test.

AChE activity was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as described in detail by Rocha et al. (1993). The assay medium contained 22 mM K+-phosphate buffer, pH 7.5, 1 mM 5,5-dithiobis-(2-nitrobenzoic) acid, 0.1 mM ethopropazine chloride (in order to inhibit butirylcholines-
terase), 0.1 or 0.8 mM acetylthiocholine iodide. After preincubation for 2 min. with 0, 10, 20, 50, 100 or 200 mM 2,5-hexanedione, the reaction was started by adding acetylthiocholine iodide and measuring the increase in absorbance at 412 nm at 25°C. The reaction was linear for up to 4 min. Enzyme specific activity was calculated as μmol acetylthiocholine hydrolysed/hr/mg protein. Data were analysed by a 3 (acetylthiocholine concentrations: 0.05, 0.1, and 0.8 mM)× 6 (2,5-hexanedione concentrations: 0, 10, 20, 50, 100, 200 mM) design with the last factor treated as repeated measure. Post hoc comparisons were made when appropriate by Duncan’s multiple range test. Protein was quantified by the method of Bradford (1976) using serum bovine albumin as standard.

Results

The effects of atropine on antinociception induced by 2,5-
hexanedione are illustrated in fig. 1. Three-way analysis of
tail-flick latency as a function of time (P<0.05).

![Fig. 1. Effect of atropine on antinociception induced in rats by 2,5-
hexanedione. After basal tail-flick measurements, animals received saline (S), 5 (A5) or 25 mg/kg (A25) atropine, intraperitoneally (pretreatment). Ten min. later, rats were injected with vehicle or 800 mg/kg 2,5-hexanedione (V or HD). Ten, 30, 60 and 90 min. after V or HD treatment, the animals were submitted to new measurements of tail-flick latencies. Data are reported as means for 14–17 rats in each experimental group. ○ S-V; ● A5-V; △ A25-V; □ S-HD; ■ A5-HD; □ A25-HD. Duncan’s multiple-range test: * P<0.05 compared to S-V (○), A5-V (●) and A25-V (△). # P<0.05 compared to all groups. The F-test for a simple effect revealed that 2,5-hexanedione-injected rats present a significant increase in tail-flick latency as a function of time (P<0.05).

Table 1. In vitro effect of 2,5-hexanedione on rat brain acetylcholinesterase activity. The enzyme was preincubated with the inhibitor for 2 min. and the reaction was started by adding acetylthiocholine, pH 7.5, at 25°C. AChE, presented as % of control activity (0 mM 2,5-hexane-
dione), is expressed as means±S.E.M: for 4 independent enzymatic
assays. Specific activity in the absence of inhibitor at 0.05, 0.1 and 0.8 mM acetylthiocholine was 0.93±0.04, 1.25±0.05 and 1.96±0.11 μmol substrate hydrolysed per hr per mg protein, respectively. Data were analysed by a two-way ANOVA (see Material and Methods for details) and yielded a significant effect of 2,5-hexanedione concentration (P<0.01) and a acetylthiocholine concentration×2,5-
hexanedione concentration interaction (P<0.01). F-test for simple effect revealed that 2,5-hexanedione inhibits significantly the acetyl-
cholinesterase hydrolysis (P<0.01). Means in the same row that do not share the same superscript are different at P<0.05 (Duncan’s multiple-range test).

<table>
<thead>
<tr>
<th>2,5-Hexanedione mM</th>
<th>Acetylthiocholine concentrations</th>
<th>% of activity</th>
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<tbody>
<tr>
<td>0.05 mM</td>
<td>0.1 mM</td>
<td>0.8 mM</td>
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<tr>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>10</td>
<td>65.7±10.3</td>
<td>73.1±7.0</td>
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<tr>
<td>20</td>
<td>41.4±3.3</td>
<td>50.0±4.2</td>
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<tr>
<td>50</td>
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<td>25.0±4.3</td>
</tr>
<tr>
<td>100</td>
<td>3.4±1.9</td>
<td>16.1±4.7*</td>
</tr>
<tr>
<td>200</td>
<td>0.0±0.0</td>
<td>6.0±3.9#</td>
</tr>
</tbody>
</table>

variance (ANOVA): 3 doses of atropine (saline (S), 5 (A5) or 25 (A25) mg/kg atropine)× 2 doses of 2,5-hexanedione (vehicle or 800 mg/kg 2,5-hexanedione)× 5 trials (basal, 10, 30, 60 and 90 min.) revealed a significant main effect of 2,5-hexanedione administration [F(1,86)=38.71, P<0.001]. ANOVA also revealed a significant 2,5-hexanedione× trials interaction [F(4, 344)=8.84, P<0.001]. The third order in-
teraction atropine×2,5-hexanedione× trials [F(8,344)=3.15, P<0.002] was also significant.

2,5-Hexanedione-treated rats showed longer tail-flick lat-
tencies than vehicle-treated rats. Analysis of the 2,5-hexane-
dione× trials interaction showed that the tail-flick latencies of rats treated with 2,5-hexanedione, but not vehicle- or atropine-injected groups, increased significantly as a function of time after drug administration. The third-order in-
teraction was significant because atropine partially reversed 2,5-hexanedione-induced analgesia. Reversion was statistically significant at 60 and 90 min. after 2,5-hexanedione administration (70 and 100 min. after atropine).

Table 1 presents the inhibitory effect of 2,5-hexanedione on rat brain acetylcholinesterase activity. Specific activity at 0.05, 0.1 and 0.8 mM acetylthiocholine iodide was 0.93±0.04, 1.25±0.05 and 1.96±0.11 μmol acetylthiocho-
line hydrolysed per hr per mg protein, respectively (data expressed as mean±S.E.M, for n=4 for each acetylthiocholine iodide concentration). The results demonstrated that AChE inhibition by 2,5-hexanedione was dose dependent (F(5,45)=258.5, P<0.01). However, 2,5-hexanedione inhibition of AChE decreased with increasing substrate concentra-
tion, as indicated by the significant acetylthiocholine concentration× 2,5-hexanedione concentration interaction (F(10,45)=3.32, P<0.01). This results suggest a competitive
type of inhibition. This is better indicated by the IC₅₀ values for AChE inhibition using 0.05, 0.1 or 0.8 mM acetylthiocholine, that were 15, 22 and 70 mM 2,5-hexanedione, respectively. The IC₅₀ was calculated by the plot of Dixon (Dixon & Webb 1964).

Discussion

The main objective of the present investigation was to determine the possible involvement of a cholinergic mechanism in analgesia induced by 2,5-hexanedione, a potent neurotoxic agent derived from n-hexane metabolism in mammals. The results clearly demonstrated that pretreatment of adult rats with the anticholinergic drug atropine considerably reduces the antinociceptive effect of 2,5-hexanedione. These data may indicate that the 2,5-hexanedione-induced analgesia has a cholinergic component (muscarinic), which is in agreement with literature data showing that inhibitors of acetylcholinesterase such as eserine and cholinergic agonist may cause antinociception in rodents (Gillberg et al. 1989; Gower 1987; Sistaram & Gillin 1977).

The possible involvement of a generalized motor impairment in 2,5-hexanedione-induced analgesia can be ruled out, because no gross sensory or motor impairment could be observed in treated rats. Furthermore, rats chronically treated with 200 mg/kg 2,5-hexanedione for 40 days showed a marked impairment in motor function but did not show increased tail-flick latencies when compared to unexposed animals (Pereira & Izquierdo, unpublished results). Thus, the observed acute analgesia caused by 2,5-hexanedione cannot be attributed to sensory or motor impairment.

Indirect evidence for the participation of inhibition of brain acetylcholinesterase in 2,5-hexanedione-induced analgesia was obtained in the present investigation. Although no detailed kinetic study was conducted, the enzyme inhibition data clearly demonstrated that a competitive component is involved in the inhibitory effect of 2,5-hexanedione on brain AChE (table 1). A direct demonstration of brain AChE inhibition after acute in vivo administration of 2,5-hexanedione is difficult for two reasons: a) sample preparation may cause a considerable dilution of the inhibitor, and 2) the lowest concentration of substrate (50 μM acetylthiocholine iodide used to accurately quantify AChE activity is at least 1 order of magnitude greater than the actual extracellular acetylcholine concentrations found in the rat brain (Fonnum & Guttormsen 1969; Lallement et al. 1992).

The pharmacological and biochemical data suggest that AChE inhibition may play a role on the analgesia observed after acute 2,5-hexanedione administration. These results are apparently at variance with data published by Bastone et al. (1987) who demonstrated that chronic exposure to n-hexane or 2,5-hexanedione increases the activity of AChE in plasma and in extracts of extensor digitorum longus muscle of rats. However, since they use a prolonged treatment, the increase in blood plasma AChE activity may represent a compensatory response to a chronic enzyme inhibition or, as suggested by Bastone et al. (1987), may be due to an increased secretion of AChE from rat muscle.

In summary, the present pharmacological data clearly support an involvement of a cholinergic component in the antinociception induced by 2,5-hexanedione, since atropine partially reverses the analgesia caused by acute administration of the neurotoxic agent. Indirect neurochemical evidence for a cholinergic involvement was also obtained, since 2,5-hexanedione inhibits brain AChE in vitro.

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References


