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Protection of acetylcholinesterase by meptazinol in mice exposed to di-isopropyl fluorophosphate. Comparison with physostigmine

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Abstract—The protective action of meptazinol against acute di-isopropyl fluorophosphate (DFP) intoxication has been evaluated in mice by measuring the effects on the DFP LD50 of the pretreatment of the animals with increasing doses of the drug. Meptazinol at the doses 15, 30 and 45 mg kg^{-1} injected 15 min before DFP caused a dose-dependent increase in the DFP LD50, resulting in protection ratios equal to 2·1, 4·8 and 9·7, respectively, in the absence of atropine and 2·5, 4·7 and 8, respectively, in the presence of atropine sulphate (17·4 mg kg^{-1}) therapy. Under the same experimental conditions, the protective ratio of 0·1 mg kg^{-1} physostigmine sulphate was 2·2 and 7·3 in the absence and presence of atropine therapy, respectively. In separate experiments, the time course of acetylcholinesterase (AChE) activity recovery was evaluated in the brain and diaphragm of mice pretreated with meptazinol (30 mg kg^{-1}) or physostigmine (0·1 mg kg^{-1}) 15 min before poisoning with DFP (8 mg kg^{-1}). Ten minutes after poisoning, residual AChE activity in the brain averaged 4, 47 and 15% of that in controls in animals pretreated with atropine alone, atropine plus meptazinol or atropine plus physostigmine, respectively. Twenty four hours after poisoning, brain AChE activity averaged 31 and 47% of that in controls in mice protected by meptazinol and physostigmine, respectively. The data from the diaphragm closely paralleled those from the brain. It is concluded that high doses of meptazinol exert antidotal action against acute DFP poisoning in the mouse comparable in efficacy with that of physostigmine combined with atropine. This action is most probably due to the ability of meptazinol to protect AChE from irreversible inactivation by DFP.

Meptazinol, (±)-m-(1-methyl-3-ethyl-hexahydro-1H-azepin-3-yl) phenol hydrochloride, is an opioid analgesic which is effective against moderate and severe pain of varying aetiologies (see Stephens et al 1978; Holmes & Ward 1985, for reviews). Besides interacting with opioid receptors, meptazinol has been shown to possess remarkable anticholinesterase activity, which is readily reversible upon dilution (Galli 1985).

Recently, we reported that meptazinol pretreatment protects mice against acute cholinesterase intoxication (Bottoncetti & Galli 1987; Galli & Mazri 1988). Therefore, meptazinol's action appears to be analogous with that of physostigmine, pyridostigmine and other carbamic anticholinesterase agents which, at low doses, exert an effective preventive action against cholinesterase poisoning by organophosphorus compounds (Berry & Davies 1970; Harris & Stitcher 1984; Deshpande et al 1986). To examine this further, in the present study we have compared the antidotal action of meptazinol with that of physostigmine against di-isopropyl fluorophosphate (DFP) poisoning in mice. At the same time we have attempted to establish a correlation between the prophylactic effects of the two drugs and acetylcholinesterase (AChE) activity.

Materials and methods

Animals. Male Swiss mice, 25–29 g, were purchased from Stefano Morini s.a.s. (S. Polo d'Enza, Italy) and housed with free access to food and water for at least 4 days before use.

Drugs and chemicals. Meptazinol hydrochloride was a gift from Wyeth S.p.A. (Rome, Italy). Physostigmine sulphate, atropine sulphate, ethopropazine hydrochloride and acetylcholine chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DFP was from Fluka AG (Buchs, Switzerland). A stock solution of DFP was prepared in propylene glycol and stored at 4°C. All drugs used in protection experiments were dissolved in 0·9% w/v NaCl (saline) and injected in a volume of 0·005 mL g^{-1}. Dilute DFP solutions were used within 15 min from the moment of preparation. [^{3}H]Acetylcholine chloride (Amersham Int. plc, Amersham, UK; 2·0 Ci $mmol^{-1}$) was mixed with unlabelled acetylcholine chloride to give 1 mCi $mmol^{-1}$ specific activity.

Measurement of protective efficacy. Randomized groups of 10 mice were treated with the protective agents or with saline alone at appropriate time intervals relative to the injection of increasing doses of DFP. When atropine was included in the treatment protocol, it was injected s.c. together with DFP. After injection of DFP, the animals were observed for 2–3 h for the onset and progress of symptoms. After this the animals were frequently, but intermittently, examined for up to 24 h. This time was used to assess overall mortality. LD50 values and 95% confidence limits were calculated according to Litchfield & Wilcoxon (1949) on the basis of the 24 h mortalities observed in groups of 10 mice given 4 to 5 increasing doses of DFP. The results of protection experiments were also expressed as the protective ratio: protective ratio = LD50 of treated animals/LD50 of untreated animals.

AChE assay. Brain and diaphragm AChE activities were measured essentially according to the radiometric method of Johnson & Russell (1975) using 2 mM [^{3}H]acetylcholine (1 mCi $mmol^{-1}$) as substrate. The final dilutions of brain and diaphragm tissues were 1–250 and 1–50 volumes, respectively. Ethopropazine (10 μM) was added to the incubation mixture to inhibit selectively butyrylcholinesterase (Silver 1974). The whole procedure, from tissue dissection to [^{3}H]acetate extraction, took approximately 30 min.

Results

The results in Table 1 show that meptazinol (15–45 mg kg^{-1}) exerted a significant dose-dependent protective effect against DFP poisoning. This effect is also documented by the protective ratios which were greater than one in meptazinol-pretreated mice. Doses of meptazinol smaller than 15 mg kg^{-1} resulted in variations in the DFP LD50 at the limit of significance (data not shown). Meptazinol given alone caused sedation in mice, and occasionally, some of the animals receiving 45 mg kg^{-1} of the drug manifested tremors and diarrhoea. No other symptoms of cholinergic activation were noticed. Meptazinol doses greater than 45 mg kg^{-1} were not tested because they were clearly toxic. The protective effect of meptazinol was evident both in the absence and presence of atropine co-administration. However, atropine treatment failed to increase the efficacy of meptazinol pretreatment, as shown by the protective ratios (Table 1). As expected, physostigmine exerted considerable protective action against DFP-induced lethality, its protective ratio being 7·3, but only in conjunction with atropine treatment. As for symptomatology, meptazinol pretreatment did not prevent surviving

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Table 1. Effect of pretreatment of mice with increasing doses of meptazinol on DFP-induced lethality in the absence and presence of atropine therapy: comparison with physostigmine.

Pretreatment[a]	Dose	Without atropine therapy LD50 (95% confidence limits) (mg kg^{-1})	Protective ratio[c]	With atropine therapy[b] LD50 (95% confidence limits) (mg kg^{-1})	Protective ratio[c]
None		4·9 (4·4–5·4)	1	7·4 (6·4–8·5)	1
Meptazinol	15	10·3 (8·5–12·5)	2·1	18 (14–23)	2·5
Meptazinol	30	23·5 (19·3–28·6)	4·8	35 (29·9–40·9)	4·7
Meptazinol	45	47·5 (39·2–57·4)	9·7	59·2 (48·9–71·6)	8
Physostigmine	0·1	10·8 (9·9–11·7)	2·2	54 (48–61)	7·3

[a] Pretreatment was carried out 15 min before DFP administration. [b] Atropine sulphate (17·4 mg kg^{-1}) was administered s.c. together with DFP. [c] Protective ratio = LD50 of treated animals/LD50 of untreated animals.

Table 2. Effect of administration time on the protective action of meptazinol and physostigmine against DFP poisoning.

	DFP LD50 (95% confidence limits) (mg kg^{-1}) Protective ratio Before DFP (min)				After DFP (s)
Treatment	120	60	30	15	30
Meptazinol	10·4 (8–12·7) 1·4	14·8 (12·5–17·8) 2·0	45·1 (38·2–53·2) 6·1	59·2 (48·9–71·7) 8·0	14 (12·0–16·4) 1·9
Physostigmine	14·8 (12–18) 2	26·6 (21–34) 3·6	40·7 (34–48) 5·5	54 (48–61) 7·3	14·8 (13–17) 2·0

Groups of 10 mice were treated with 45 mg kg^{-1} meptazinol or 0·1 mg kg^{-1} physostigmine 120, 60, 30 and 15 min before or 30 s after the administration of scalar doses of DFP together with atropine sulphate (17·4 mg kg^{-1}). Protective ratio = LD50 of treated animals/LD50 of untreated animals.

animals from showing severe symptoms of cholinesterase intoxication, but these animals recovered within 24 h. Physostigmine pretreatment generally resulted in faster recovery times (4–6 h).

In separate experiments, the time course of the protection by meptazinol and physostigmine was evaluated by pretreating groups of mice with fixed doses of the two drugs (45 and 0·1 mg kg^{-1} respectively) at different times before (120, 60, 30 and 15 min) or after (30 s) DFP poisoning. Atropine sulphate (17·4 mg kg^{-1}) was given to all animals together with DFP. As shown in Table 2, the protective effects of the two drugs were markedly affected by the time of drug administration relative to DFP poisoning.

Fig. 1 shows the effect of pretreatment with protective doses of meptazinol (30 mg kg^{-1}) and physostigmine (0·1 mg kg^{-1}) on brain and diaphragm AChE activity in mice poisoned with a fixed dose (8 mg kg^{-1}) of DFP. Atropine sulphate (17·4 mg kg^{-1}) was administered to all animals together with the protective agents. Under these conditions all the animals pretreated with meptazinol and physostigmine recovered during 24 h. Most of those treated with atropine and DFP alone, that is without protection, died during the first hour after poisoning and for this reason Fig. 1 reports the values obtained with these animals only at 10 min after DFP. The results of Fig. 1 show that in unprotected animals, DFP poisoning caused brain AChE activity to decline from 100% to 4 ± 1% of that in controls, while the fraction of free active AChE was significantly ($P < 0·001$) higher in animals pretreated with meptazinol (47 ± 5%) or physostigmine (15 ± 1%). During the next 10–60 min, AChE activity kept on declining in meptazinol-pretreated animals (22% vs 47%; $P < 0·001$), while it was essentially unmodified in the physostigmine group (18% vs 15%). The recovery of enzymic activity was more rapid in the physostigmine than in the meptazinol group. In fact, at the end of the observation period, brain AChE was significantly higher in physostigmine- than in meptazinol-pretreated mice (47 ± 4% vs 31 ± 2% of controls, respectively; $P < 0·001$). The data from the diaphragm (Fig. 1) basically paralleled those from the brain, but the recovery of AChE activity was slightly higher than in brain. Pretreatment of 6 mice with atropine and meptazinol only (meptazinol controls), that is without DFP poisoning, resulted in AChE activities not significantly different from atropine controls, whereas physostigmine controls (6 mice) showed brain and diaphragm AChE

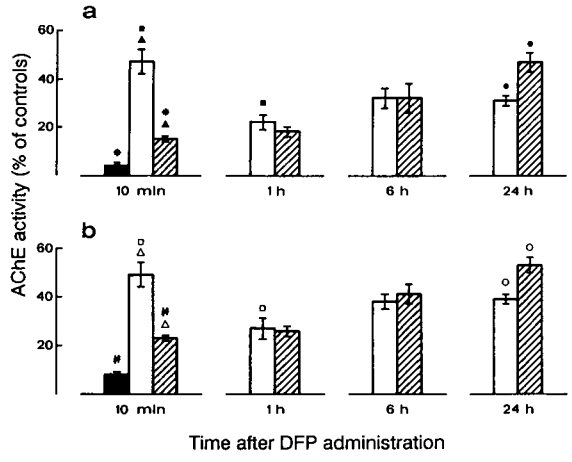

FIG. 1. Time course of brain (a) and diaphragm (b) AChE activity in mice poisoned with DFP: effect of pretreatment with meptazinol and physostigmine. Groups of mice were pretreated with atropine sulphate (17·4 mg kg^{-1} s.c., filled) alone or atropine sulphate plus meptazinol (30 mg kg^{-1} s.c., open) or atropine sulphate plus physostigmine (0·1 mg kg^{-1} i.p., hatched) before treatment with DFP (8 mg kg^{-1} s.c.). The results are expressed as percent of the mean brain (8·5 ± 0·4 μmol g^{-1} min^{-1}) and diaphragm (0·88 ± 0·1 μmol g^{-1} min^{-1}) AChE activities observed in 9 mice treated with atropine and saline alone (controls). Bars and vertical lines represent the mean + s.e. of 9 animals. Values sharing the same symbol are significantly different (Student's t-test for grouped data) at the following levels: △, ▲, *, #, = $P < 0·001$; ○, □, ●, ■ = $P < 0·01$.

activities which averaged 44, 82 and 95% of that in atropine controls measured at 25 min, at 75 min and 6·125 h, respectively, after physostigmine. Therefore, in the poisoned animals pretreated with physostigmine, a substantial fraction of AChE was, at least during the first hour after poisoning, blocked by physostigmine itself.

Discussion

The results of this work, taken together with previous data from this laboratory (Galli & Mazri 1988), provide evidence that the opioid analgesic meptazinol is effective in protecting mice against DFP-induced lethality. In this study, the efficacy of meptazinol's antidotal action has been quantified by evaluating its protective ratio under different experimental conditions.

The observation that the protective action of meptazinol does not require atropine therapy is a novel finding in this study. Meptazinol markedly differs from physostigmine and other carbamic protective agents, which exert an effective protective action only when administered in conjunction with anticholinergic drugs (Berry & Davies 1970; Harris & Stitcher 1984; Deshpande et al 1986). The characteristics of meptazinol's action suggest an analogy between this drug and eseroline which also is effective as an antidote against DFP in the absence of anticholinergic treatment (Galli et al 1985). Atropine therapy does not increase the meptazinol protective ratio, but it does cause a generalized increase in the DFP LD50 values. Therefore, the maximal dose of meptazinol tested (45 mg kg^{-1}) was more effective than the standard protective dose (0·1 mg kg^{-1}, Gordon et al 1978) of physostigmine in the absence of atropine while the two drug doses were approximately equipotent when administered in conjunction with anticholinergic therapy. Under these conditions, meptazinol is about 450 times less potent than physostigmine, but is practically devoid of cholinomimetic effects (Stephens et al 1978) and therefore can be administered at relatively high doses without problems.

Meptazinol at the doses which are effective in the prophylaxis against DFP poisoning protects central and peripheral AChE from the irreversible inactivation caused by this agent. We had previously reported that meptazinol protects mice against physostigmine, but not against the quaternary ammonium derivative neostigmine (Bottoncetti & Galli 1987). Since neostigmine does not cross the blood-brain barrier appreciably (Rosecrans & Domino 1974), we assumed that meptazinol exerts its protective action mainly on AChE in the central nervous system (Bottoncetti & Galli 1987). However, the results of our present study indicate that such a hypothesis is probably incorrect. Meptazinol appears to be effective, at least against DFP, on peripheral as well as central AChE. The inefficacy of meptazinol against neostigmine-induced lethality remains to be explained. It appears likely, however, that other mechanism(s), in addition to AChE inhibition, may be involved in neostigmine toxic effects. In this respect, it appears of interest to point out that quaternary anticholinesterase agents exert a direct action on skeletal muscles which is unrelated to AChE inhibition (Taylor 1985).

When AChE is examined 10 min after poisoning, the moment critical for survival, meptazinol is much more effective than physostigmine in protecting the enzyme. This may explain why in meptazinol-pretreated mice, the atropine requirement is not as important as in those protected by physostigmine. The higher protective ratio of physostigmine in comparison with meptazinol at the drug doses used in these experiments may be explained by the overall AChE recovery at the end of the observation period which was significantly higher in the physostigmine than in the meptazinol group.

These results appear to support the hypothesis that the primary mechanism of the antidotal action of meptazinol is represented by its ability to prevent irreversible inhibition of AChE by DFP (Green 1983; Lennox et al 1985). Meptazinol, lacking a reactive acylating group, cannot steadily block AChE. Its protective action, therefore, is likely to take place on the formation of the reversible enzyme-inhibitor complex (Main 1979). Meptazinol might prevent DFP attack by shielding the active site of AChE from the organophosphorus agent. This mechanism of action has been described before to explain the protective actions of other reversible anti-cholinesterase agents (Harris et al 1978; Galli et al 1985). However, our data cannot completely rule out the possibility that mechanism(s) other than AChE protection (Kawabuchi et al 1988) may be involved in the antidotal action of meptazinol.

This work has demonstrated that meptazinol at relatively high doses exerts prophylactic action against acute DFP poisoning in the mouse which is comparable in efficacy to that of a standard protective dose of physostigmine combined with atropine. This antidotal action of meptazinol is most probably due to the drug's ability to protect central and peripheral AChE from irreversible inactivation by DFP. The meptazinol analogues with a high affinity for AChE show promise as new prophylactic agents potentially effective against organophosphorus poisoning.

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The effect of nitrous oxide on *S*-adenosylmethionine levels in mouse brain

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Abstract—Mice were exposed to nitrous oxide (50%) for up to 24 h and *S*-adenosylmethionine (SAMe) levels measured in corpus striatum and cerebellum, areas with high and low catecholamine turnover rates, respectively. After 4 h, levels were 21 and 8% and after 6 h, 33 and 14% lower than controls in striatum and cerebellum, respectively. Thus, the effect was more pronounced in corpus striatum, the area with the presumed higher rate of catecholamine *O*-methylation. With continued exposure to nitrous oxide SAMe concentrations in the two areas returned to nearly normal at 24 h. The observation that levels did not continue to decline, and even returned towards control levels, while animals were still in the presence of the gas suggests that a mechanism other than that of methionine synthase inhibition may have been responsible for the initial effect. Alternatively, some other source of SAMe may have become available to compensate for the inhibition of the enzyme.

Although methionine is an essential amino acid, an interference with the contribution provided by the enzymatic conversion of homocysteine to methionine by methionine synthase (EC 2.1.1.13) can result in a decrease in tissue levels of the amino acid. Thus, nitrous oxide (N_2O) exposure has been shown to decrease the activity of methionine synthase in rodents and result in diminished tissue levels of methionine (Koblin et al 1981; Lumb et al 1983; Brennt & Smith 1989). The enzyme requires cobalamin I (vitamin B_{12}) as a cofactor and the mechanism of its inhibition by N_2O is thought to result from oxidation of cobalamin I to cobalamin III, a form of the coenzyme that is inactive (Banks et al 1968).

S-Adenosylmethionine (SAMe) is derived from methionine and exposure to N_2O in some studies has resulted in its diminution in liver (Lumb et al 1983) and whole brain (Vina et al 1986).

SAMe is a methyl donor in a variety of transmethylation reactions, including the *O*-methylation of the catechol neurotransmitters. Under normal circumstances, the biosynthesis of SAMe keeps pace with its utilization. However, there is evidence that demand for the methyl donor can outstrip its supply. For example, rats treated for 1 h with haloperidol, a dopamine receptor blocking drug that is known to reflexly increase dopamine turnover in the dopamine-rich corpus striatum, had approximately a 30% decrease in SAMe levels in that brain area (Waldmeier & Feldtrauer 1987). Thus it seemed possible that, since N_2O can inhibit methionine synthesis, and even possibly lower whole brain SAMe levels, the agent might have a greater effect on SAMe levels in corpus striatum than in cerebellum, an area with relatively little catecholamine content (Heffner et al 1980). This possibility was investigated.

Materials and methods

Male mice, 30–40 g, HSD: (ICR) BR were exposed to $N_2O:O_2$ using 4 L filtration flasks as exposure chambers and a Quanitiflex MDM, N_2O machine. The gas mixture (50:50) was delivered at 4 L min^{-1} via a glass tubing which passed through a rubber stopper in the opening of each flask and extended to within 5 cm of the bottom. Gases exited via the flask side arm and were vented into a fume hood. Two flasks, each containing two mice, received the gases simultaneously by using a split connector in the line. Two other flasks were similarly arranged but with $N_2:O_2$ (50:50) serving as control gases.

The animals were killed by exsanguination under chloroform or ether anaesthesia. Brains were chilled in cold saline and placed on a chilled plate for dissection of corpus striatum and a portion of cerebellum approximating the weight of the corpus striatum. Tissue SAMe concentrations were determined using a method based on that of Wagner et al (1984). This method has a detection limit of approximately 1 pmol which far exceeds the sensitivity needed in the present study. Thus, after tissues were blotted and weighed, they were homogenized in 1 mL 0·2 M perchloric acid and centrifuged at 4°C. The supernatants were filtered through 0·2 μm cellulose filters in a centrifugal filtering apparatus. Portions of the filtrates were subjected to HPLC, and absorbance was measured at 254 nm. The HPLC mobile phase consisted of 850 mL 0·15 M NaH_2PO_2, 150 mL acetonitrile and 1·73 g sodium octanesulphonic acid. The pH was adjusted to 3·0 with 3 M phosphoric acid. Early in the study it was noticed that absolute control values seemed to vary (though not consistently) with the time of day. For that reason, controls were always included in each experiment and results expressed as percentage change from these controls. Statistical differences between means were determined by Tukey's test.