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ORIGINAL ARTICLE

Is a high dose of Huperzine A really suitable for pretreatment against high doses of soman?

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Summary
Huperzine A (Hup A) is a reversible AChE inhibitor that crosses the blood-brain barrier. It is presently approved for, or is in a course of clinical trials for, the treatment of Alzheimer's disease. This compound has also been successfully tested for the pretreatment of organophosphate poisoning. Organophosphate nerve agents are potent irreversible inhibitors of acetylcholinesterase in the central and also in the peripheral compartment. In this study Hup A in a higher dose (500 μg/kg) was tested as a prophylaxis against a high, mainly centrally acting, nerve agent (soman). According to the results obtained, Hup A in this dosage was not able to protect AChE against soman in both the peripheral and central compartments. The effect of Hup A and soman was found to be additive and all animal subjects died.

Key words: Huperzine A; soman; brain; nerve agent; acetylcholinesterase, pretreatment

INTRODUCTION

The treatment of intoxication caused by highly toxic organophosphates (OPs) such as soman is still very complicated because there is no satisfactory therapy for the effects of these compounds, especially soman. Soman is a high lipophilic which possesses a strong ability to pass through the blood-brain barrier (Andersson et al. 1992). OPs act as potent irreversible inhibitors of cholinesterases (ChE). The main mechanism of their action is interaction with acetylcholinesterase (AChE; 3.1.1.7) and subsequent accumulation of neurotransmitter acetylcholin (ACh) in the synapses, the central nervous system and neuromuscular junctions. This ACh imbalance induces disturbance of numerous body functions such as salivation, lacrimation, respiratory distress, muscle fasciculation and generalized seizures (Holmstedt 1959).

The standard treatment for such poisoning is the administration of anticholinergic drugs (e.g. atropine) which antagonize the effects of acetylcholine accumulated at the cholinergic synapses, and AChE reactivators (oximes) which reactivate inhibited AChE. Their effects are synergistic (Bajgar 2004). The standard treatment is practically ineffective after

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intoxication caused by soman, because its main action is in the central nervous system. The blood-brain barrier is practically impermeable for atropine and AChE reactivators. The second reason for the low efficacy of standard treatment is the rapid "ageing" of the soman-inhibited AChE (Bajgar et al. 2007a).

Another strategy in therapy for OPs intoxication includes the use of AChE protection by prophylaxis (Patočka et al. 2006) The term prophylaxis means that medical countermeasures are applied a relatively short time before penetration of a toxic agent into the organism (Bajgar et al. 2007b). One example is the protection of AChE against nerve agent inhibition using reversible cholinesterase inhibitors as prophylaxis (Aldridge 1969).

Among the reversible inhibitors the most interesting results have been obtained with Huperzine A (Hup A) (Bajgar et al. 2007b). Hup A, an alkaloid isolated from *Huperzia serrata*, is a powerful and reversible inhibitor of AChE both at the peripheral and central compartments (Zangara 2003). Its potency to inhibit AChE is similar or superior to that of physostigmine, galanthamine or tacrine (Gunwald et al. 1994, Zhao et al. 2002, Eckert et al. 2007). Hup A is also a powerful neuroprotective and antioxidant agent, because it can reduce neuronal cell death caused by an excess of glutamate (Filliat et al. 2002). This action enhances the potential value of this compound as a prophylactic and therapeutic agent for intoxication of OPs.

In this study, we wanted to test the effect of a higher dose of Hup A as a prophylactic drug against soman.

MATERIALS AND METHODS

Chemicals

The nerve agent (soman) of approximately 98% purity was obtained from the Military Technical Institute of Protection (Brno, Czech Republic). Hup A was purchased from Sigma Aldrich (Prague, Czech Republic).

Animals

The whole *in vivo* experiment was conducted using Wistar albino male rats. The animals were maintained in an air-conditioned room, and were allowed free access to standard chow and tap water. The experiment was performed with the permission of and under the supervision of the Ethics Committee of the Medical Faculty of Charles University and the Faculty of Military Health Sciences, Hradec Králové.

Intoxication and treatment

A single dose of 500 µg/kg Hup A was administered i.p. 60 min before intoxication by single doses of 1.5 LD_{50} of soman (80 µg/kg; i.m). The animals were killed by decapitation 30 min after the nerve agent intoxication.

Biochemical determination of AChE

After decapitation, the brain, diaphragm and blood were withdrawn. The organs were then frozen and stored at –80 °C. Immediately after thawing, tissues were homogenized (1:10, 0.02M Tris-HCl buffer, pH 7.6; DI 25 Homogenizer, IKA-WERKE, Germany) and the homogenates were used for enzymatic analysis.

The activities of AChE were assessed by Ellman's method with acetylthiocholine iodide as substrates and 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB, $\varepsilon = 14.15 \times 10^3\ M^{-1}\ cm^{-1}$) as chromogen (Ellman et al. 1961). A Helios Alpha spectrophotometer (Electron Corporation, Great Britain) was used for the determination of absorbancy at wavelength 436 nm and the results were expressed as µcat/g wet weight tissue (Figs 1–4). The wavelength 436 nm was chosen because, when using the standard wavelength 412 nm. the results are effected by interaction with haemoglobin.

The activity of AChE was determined according to the usual method of calculating the accumulated DTNB after incubation for one minute. For determination we used Lambert-Beer law, where the change in enzyme activity is expressed by the equation:

$$v = \frac{\Delta A}{\varepsilon \times l \times t}$$

where ΔA is the change in absorbance during measurement, ε is the molar coefficient of absorbancy, l is the width of the cuvette and t is the time interval of the measurement in seconds.

Statistical evaluation

Enzyme activities were expressed as a mean ±SD or % of control values (means only) and statistical differences were tested by the t-test. The results of the tested groups were compared with the results of controls. We used the significance level $2\alpha=0.05$.

RESULTS

Keeping AChE intact is a basic requirement for effective prophylaxis. Reversible inhibitors protect

part of AChE (optimally 30% of AChE activity in whole blood), and they change the enzyme in a way that makes it resistant to subsequent OPs inhibition (Bajgar 2004). Reversible inhibitors are able to spontaneously recover AChE activity after a certain time interval. Carbamates are the most commonly used reversible inhibitors (Aldridge 1969), and their ability to protect an organism poisoned with OPs has been known for many years (Koelle 1946, Koster 1946). Inhibitors structurally different from the carbamates and OPs groups have also been studied intensively for their protective effect, and compounds such as tacrine and its derivatives or Huperzine A have been studied for the same purpose (Fusek 1977, Bajgar et al. 1983, Ashani 1992, Patočka 1998, Lallement et al. 2002a).

Measurement of AChE activity was carried out using the most sensitive and commonly used method as described by Ellman et al. (1961). The results of AChE activity in whole blood are summarized in Fig 1. Although the dose of Hup A evoked depression of AChE activity in whole blood, there were no significant differences between activity at 60 min or 90 min after Hup A administration. Although Hup A was administered 60 min before soman intoxication, this subsequently administered nerve agent caused strong inhibition of AChE in whole blood after intoxication. All animals (100%) from the group intoxicated by soman died before the 30 min limit. The first deaths of rats were observed approximately 10 minutes after soman intoxication. Before their death the typical signs of OPs intoxication were observed; the most typical were salivation and muscle fasciculation. In these cases, the blood and organs were withdrawn immediately the animals died. These typical signs for AChE inhibition were found after Hup A (500 μg/kg) administration, before intoxication by nerve agents. The most manifested signs were salivation, lacrimation and mild tremor.

The changes of AChE activity in the diaphragm, the most interesting peripheral tissue because of depressed breathing, were also measured. Soman caused a strong decrease in AChE levels and this instance of AChE activity corresponded with the results in whole blood. The changes of AChE activity in the diaphragm are summarized in Fig. 2.

Changes in AChE activity were also recorded in the brain (Fig. 3). From the many structural parts of the brain we chose the pontomedullar area and basal ganglia. These structures are important for the study of the distribution of inhibitors of AChE in the central nervous system. The figure shows the average control values.

Special importance can be assigned to the pontomedullar area, due to its important role in respiration. Depression of the central respiratory control centres in the pontomedullar area is considered as a primary event leading to death (Cheng and Tang 1998, Kubín and Fenik 2004). It is also known that the survival of intoxicated animals correlates with AChE activity in the pontomedullar area (Bajgar et al. 2007a). This fact was confirmed in our study: all animals intoxicated by soman died before the 30 min time interval and a strong depression of AChE activity in the pontomedullar area was recorded after soman intoxication.

Basal ganglia were the second part of the brain studied. Mammalian basal ganglia are associated with a variety of functions: motor control, cognition, emotions, and learning. In the basal ganglia, very high AChE activity is typical (Cheng and Tang 1998).

In our study, we recorded a reduction of AChE activity in the basal ganglia after Hup A administration (results in Fig. 4). There was a significant difference in AChE activity in the time intervals 60 and 90 min. The depression of AChE activity was approximately 30% after 60 min, and approximately 20% after 30 min compared to the control group. The highest difference was achieved after intoxication by soman.

In other studies, it was found that Hup A crosses the blood-brain barrier (Cheng and Tang 1998). This fact was confirmed in our study because of strong AChE inhibition in both brain parts. Time-dependent progressive brain AChE inhibition was observed in both central compartments assessed. Surprisingly, very strong inhibition was recorded also in the basal ganglia.

DISCUSSION

Some studies have been published which discuss the possibility of the use of Hup A in protection of AChE activity against soman inhibition. Lallement et al. (1997) found in their work, that Hup A totally prevented seizures and ensured the survival of all animals for 24 hours after soman intoxication. This compound was able to protect the hippocampal tissue of guinea pigs against neuronal damage. The same author in other work (Lallement et al. 2002b) found evidence that subchronic Hup A pre-treatment of primates gives them better tolerance to the epileptic effect of soman.

Tondulli et al. (2001) confirmed the results of Lallement et al. (1997) and found evidence that only a high dose of Hup A (500 μg/kg) gave protection against soman. Indeed, Hup A at a lower dose (100 μg/kg) did not prevent the occurrence of

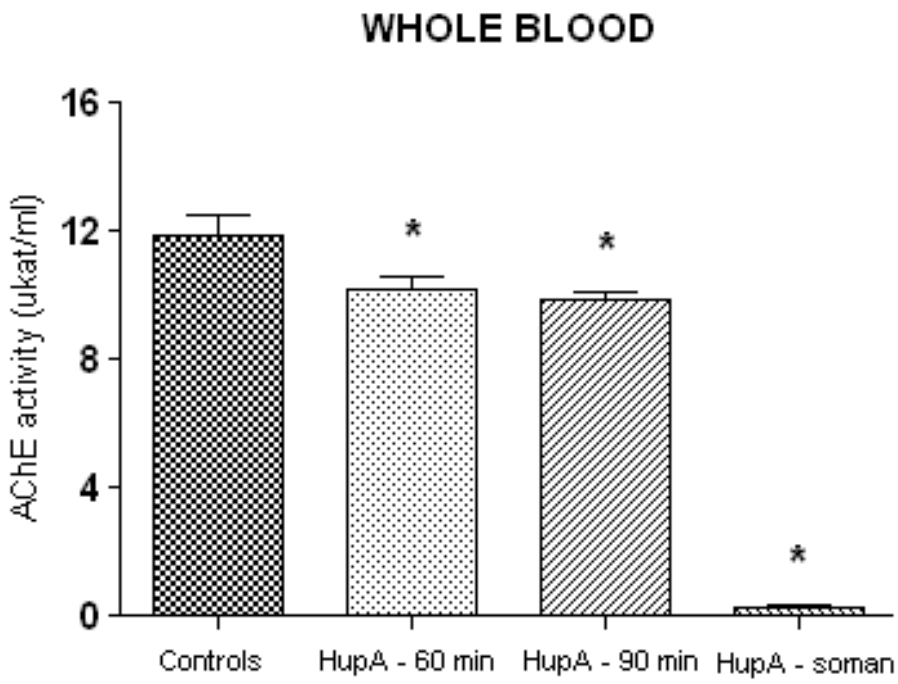

Fig. 1. **The changes of AChE activities in whole blood after administration of Huperzine A and soman.** Statistical differences we tested by t-test. *Statistically significant as compared with controls.

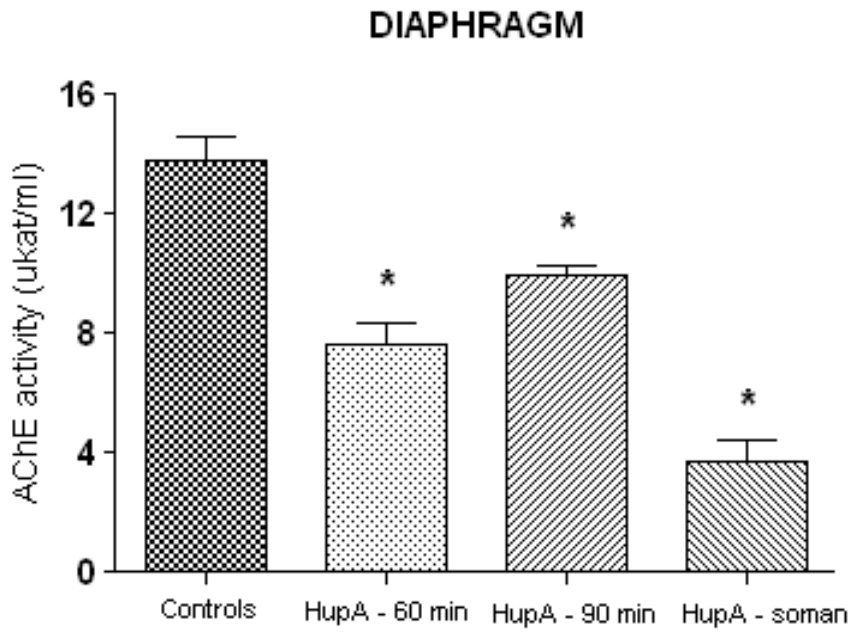

Fig. 2. **The changes of AChE activities in peripheral tissue (diaphragm) after administration of Huperzine A and soman.** Statistical method and significance as in Fig. 1.

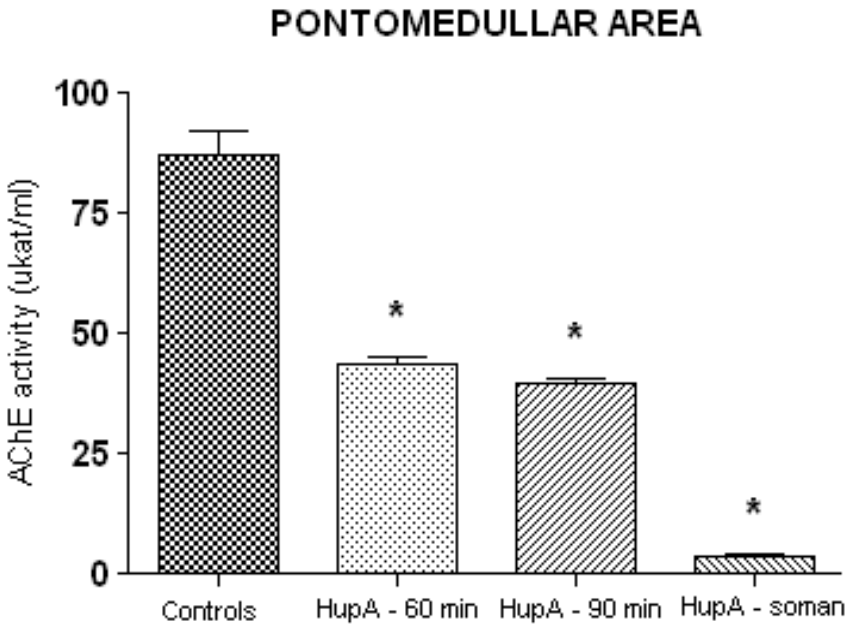

Fig. 3. **The changes of AChE activity in brain (pontomedullar area) after administration of Huperzine A and soman.** Statistical method and significance as in Fig. 1.

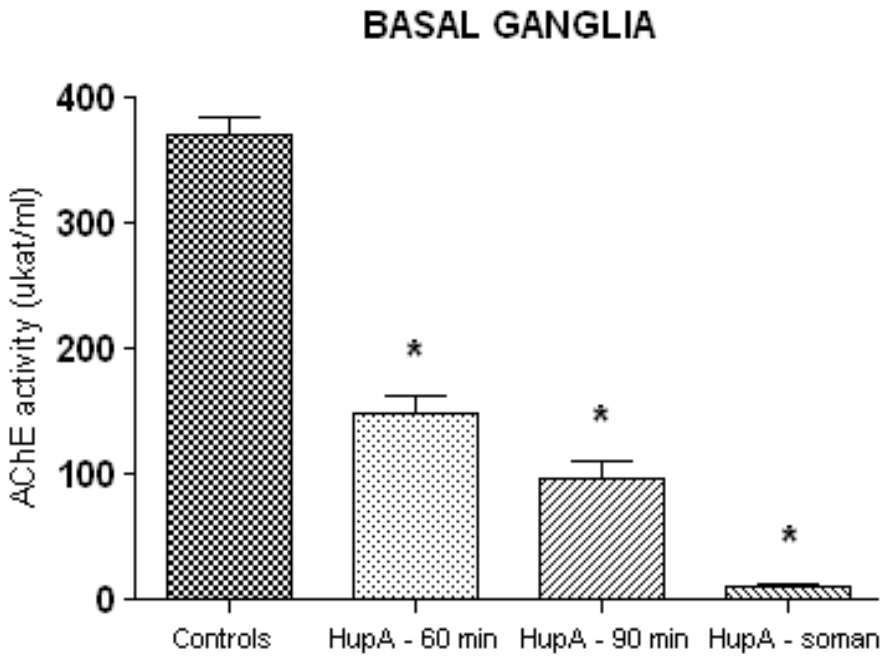

Fig. 4. **The changes of AChE activity in brain (basal ganglia) after administration of Huperzine A and soman.** Statistical method and significance as in Fig. 1.

seizures. Pre-treatment with Hup A (irrespective of the dose) was effective in reducing lethality after soman poisoning.

These results were not confirmed in our study. On the contrary, we observed a low effectiveness of a high dose of Hup A against soman poisoning. After administration of Hup A (500 μg/kg) visible signs of intoxication were evoked, which were caused by peripheral and central AChE inhibitions. The subsequent administration of another strong inhibitor (in this case soman) ended in the death of the intoxicated organism. It is clear that this reversible inhibitor (Hup A) in a relatively high dose had an additive effect on soman inhibition, and this resulted in the death of the experimental animals.

CONCLUSIONS

In conclusion, a high single dose of (500 μg/kg) Hup A was not able to protect AChE in the peripheral as well as the central compartment against death caused by soman. The inhibition efficacy of the reversible inhibitor (Hup A) administered alone in this dose was really high in the central nervous system. The reactivation of reversible inhibited AChE was slower than we expected. The cholinesterase inhibition of both inhibitors had an additive character.

The data achieved here seem to indicate that Hup A could be a useful prophylaxis reagent. However, these results need to be clarified by further experimentation.

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