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The bisphosphonates HEBP and AHPrBP but not AHBP inhibit mineral mobilization and lysosomal enzyme release from mouse calvarial bones in tissue culture[1]

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Summary. Mice injected with DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine], a selective noradrenergic neurotoxin, had marked depletions of central noradrenaline and an attenuated post-decapitation reflex. DSP-4-treated mice exhibited an increased sensitivity to the α_2-adrenoceptor agonist clonidine as measured by inhibition of the pinna reflex, but normal sensitivity as measured by hypothermia. This differential sensitivity may reflect the presence of supersensitive postsynaptic α_2-adrenoceptors in some, but not all, CNS regions after DSP-4 treatment.
Key words. DSP-4; noradrenaline depletion; α_2-adrenoceptor; clonidine; pinna reflex; oesophageal temperature; mouse.

A peripheral injection of DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine] causes depletion of central noradrenaline (NA) in rodents[1]. This depletion has been shown by recent neurochemical and behavioral experiments to induce central α_2-adrenoceptor supersensitivity[2,3].

In the present study, we investigated whether or not the α_2-adrenoceptor influence on 2 physiologic variables, pinna reflex and body temperature, would be altered in mice by DSP-4. Both variables are sensitive to α_2-adrenoceptor activation by the α_2-agonist clonidine: the pinna reflex is inhibited[4] and body temperature is decreased[5,6]. The post-decapitation reflex (PDR) was also assessed in DSP-4 mice since recent reports indicate that this reflex is abolished in DSP-4 rats[2,7-9].

Materials and methods. Male mice (CD-1, Charles River, Manston, Kent, England), weighing 20–24 g, were injected i.p. with DSP-4 hydrochloride (75 mg/kg) or the control solution (0.9% saline) in a volume of 0.01 ml/g. They were then maintained under standard laboratory conditions (20 ± 1 °C, 12-h light-dark cycles, free access to food and water) for 10 days. At 10 days, the control and DSP-4 mice were divided into 2 groups and used in experiments.

The first group of mice was used to assess the PDR and the magnitude of NA depletion in several CNS regions (e.g., neocortex, hippocampal formation, hypothalamus, cerebellum, and spinal cord). For determining changes in the PDR, the latency to convulse and duration of the tonic-clonic convulsions were measured. The NA concentrations were measured using a fluorometric method[10]. Results were analyzed using the t-statistic for group means. The minimal level of significance was $p \leq 0.01$ (2-tail criterion).

The second group of mice was administered clonidine hydrochloride to determine possible alterations in the sensitivity of the pinna reflex and oesophageal temperature. For assessment of the pinna reflex, groups of 10 mice were injected with different doses of clonidine. The presence or absence of the reflex was determined 20 min later by stimulating each auditory meatus with a fine wire. If stimulation of either ear elicited a rapid twitch of the head, the pinna reflex was deemed present. The percentage inhibition of this reflex, based on the ratio of the number of animals showing reflex inhibition to the number of animals tested, was calculated for each dose of clonidine; ED_{50} values and 95% confidence intervals (C.I.) were subsequently obtained by probit analysis[11], and lines and ED_{50} values were compared[11]. Control mice were also tested with p-aminoclonidine, a peripherally-acting α_2-agonist[3]. For measurement of oesophageal temperature, groups of 5 mice were placed in observation cages mounted on a raised grid which facilitated air circulation (ambient temperature = 22 ± 1 °C). Saline or clonidine was injected, and oesophageal temperature was taken after 30 min using a digital thermometer (Type 3009 Ni-Cr/Ni-Al, Comark Electronics Ltd., Rustington, Sussex, England). (Preliminary experiments indicated that the hypothermic effect of clonidine was maximal at the 30-min timepoint.) Results were analyzed using 2-way analysis of variance followed by Dunnett's multiple comparison statistic (2-tail criterion, $p \leq 0.01$). Control mice were also tested with p-aminoclonidine.

Results. DSP-4 treatment produced marked reductions of NA ($\leq 25\%$ of control NA remaining) in all CNS regions examined (table), the exception being the hypothalamus in which there was only a trend towards NA depletion (77% of control NA remaining). The assessment of the PDR indicated significant differences ($p \leq 0.01$) between control and DSP-4 mice as regards both latency ($\bar{X} \pm SE$, $n = 8$: 6.1 ± 0.7 and 14.5 ± 1.7 sec, respectively) and duration (15.8 ± 0.9 and 9.0 ± 0.7 sec, respectively).

Clonidine inhibited the pinna reflex in both control and DSP-4 mice (fig. 1); however, the ED_{50} for DSP-4 animals was ~2

times lower than that for control mice. p-Aminoclonidine also inhibited the pinna reflex in control mice, but the ED_{50} was ~ 9 times greater than that of clonidine (fig. 1, legend).

Clonidine caused hypothermia in both control and DSP-4 mice (fig. 2). This effect ranged between 0.5–2.5 °C, and was generally of similar magnitude for both groups across all doses tested. p-Aminoclonidine was ineffective in eliciting hypothermia at molar doses which approximated those of clonidine having an effect (fig. 2, legend).

Discussion. Consistent with other findings in rodents[1,2], DSP-4 markedly depleted NA in several CNS regions (e.g., neocortex, cerebellum, spinal cord) but not hypothalamus. The insensitivity of hypothalamus to the action of DSP-4 may reflect the mixed noradrenergic input to this region[1]; the effect of DSP-4 is greatest in regions innervated by noradrenergic neurons originating solely or mainly from the locus ceruleus.

The PDR in DSP-4 mice was characterized by a slower onset and shorter duration as compared to that in control animals. In contrast, the PDR in DSP-4 rats is essentially absent[2,7–9]. All of these changes in the rodent PDR are assumed to be a consequence of NA depletion in the spinal cord. The presence of an intact, though attenuated, PDR in DSP-4 mice, however, may indicate the importance of other neurotransmitters (e.g., dopamine and serotonin)[12] in maintaining the reflex in this species. Nevertheless, the altered PDR can be used as a screen for an effective or ineffective noradrenergic lesion after DSP-4 treatment.

The pinna reflex in DSP-4 mice was more sensitive to inhibition by clonidine than that in control mice. A logical explanation for this shift of the dose-effect relationship is the presence of supersensitive α_2-adrenoceptors. Specifically, 1) DSP-4 destroys noradrenergic neurons descending from the locus ceruleus to the spinal cord, 2) depletion of spinal NA induces α_2-adrenoceptor supersensitivity on cervical perikarya which are involved in the pinna reflex, and 3) clonidine activates these postsynaptic α_2-adrenoceptors to inhibit the reflex. This interpretation of supersensitive postsynaptic α_2-adrenoceptors is consistent with conclusions of previous neurochemical and behavioral studies[2,3]. A central location of the α_2-adrenoceptors was confirmed by the relatively weak potency of p-aminoclonidine.

In contrast to the increased sensitivity of the pinna reflex to clonidine, the magnitude of the hypothermic effect produced by this drug was the same in both control and DSP-4 mice. This suggests that the α_2-adrenoceptors mediating hypothermia are unaltered due to sufficient activation by remaining NA. These α_2-adrenoceptors are centrally located since p-aminoclonidine, at comparable molar doses to those of clonidine, was ineffective in causing hypothermia. Their location is most likely the hypothalamus: lesioning the ventral noradrenergic bundle (and markedly reducing hypothalamic NA) enhances the hypothermic response to clonidine[13]. As stated above, the hypothalamus is relatively insensitive to the NA-depleting action of DSP-4, which may explain the observation of normal clonidine-induced hypothermia.

An interesting implication of this study is that clonidine-induced inhibition of the pinna reflex may serve as a simple model for assessing the functional state of central postsynaptic α_2-adrenoceptors after various physiologic and pharmacologic manipulations.

Noradrenaline concentrations in discrete regions of the mouse CNS 10 days after DSP-4 treatment

Region	Control (nmol/g tissue)	DSP-4 (nmol/g tissue)	Percentage of control
Neocortex	1.19 ± 0.11	≤ 0.30	≤ 25
Hippocampal Formation	3.97 ± 0.27	≤ 0.30	≤ 8
Hypothalamus	13.83 ± 1.33	10.67 ± 2.11	77
Cerebellum	2.00 ± 0.21	≤ 0.30	≤ 15
Spinal Cord	2.66 ± 0.17	≤ 0.30	≤ 11

Values given, unless otherwise stated, are $\bar{X} \pm SE$, n = 8. The values for DSP-4, excluding hypothalamus, were at or below the sensitivity limit of the fluorometric method (0.30 nmol/g = 50 ng/g). Consequently, these values were not amenable to statistical analysis. The effect of DSP-4 on the hypothalamus was not significantly different from that of the control treatment.

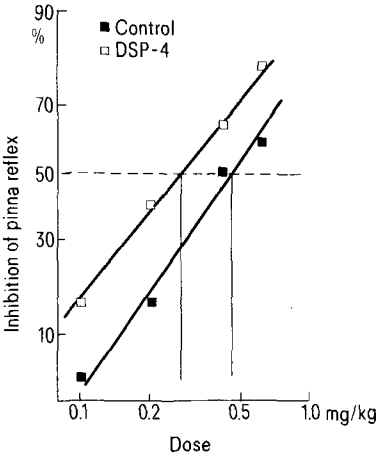

Figure 1. Log dose-log effect relationships of clonidine-induced inhibition of the pinna reflex in mice. Mice were injected with clonidine (0.1–0.6 mg/kg i.p.) 10 days after DSP-4 treatment. Each value is based on results pooled from 2 separate experiments each with n = 10 per dose. The ED_{50} (95% C.I.) for Control was 0.45 (0.33–0.62) mg/kg, and that for DSP-4 was 0.27 (0.20–0.37) mg/kg. The 2 lines did not deviate significantly from parallelism. There was a significant difference between the ED_{50} values, and the potency ratio (95% C.I.) was 1.7 (1.1–2.6). With reference to this figure, the response of control mice to p-aminoclonidine (1.25–10 mg/kg i.p.) was also tested. The ED_{50} (95% C.I.) of the drug, based on a single experiment with n = 10 per dose, was 4.2 (2.5–6.9) mg/kg. A comparison of the lines for clonidine and p-aminoclonidine (not shown) indicated no significant deviation from parallelism. The potency ratio (95% C.I.) was 9.34 (5.1–17).

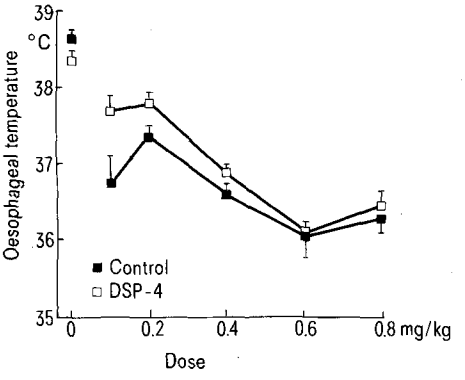

Figure 2. Dose-effect relationships of clonidine-induced hypothermia in mice. Mice were injected with clonidine (0.1–0.8 mg/kg i.p.) 10 days after DSP-4 treatment. Values given are $\bar{X} \pm SE$, n = 10. The treatment condition (Control or DSP-4) did not alter the response to clonidine [$F(1,108) = 0.60$, NS]. Clonidine produced hypothermia [$F(5,108) = 8.16$, $p < 0.01$] which was significant ($p \leq 0.01$) at all doses as compared with saline injection. There was no significant interaction between treatment condition and dose of clonidine [$F(5,108) = 0.50$, NS]. With reference to this figure, the response of control mice to p-aminoclonidine (0.2 and 0.6 mg/kg i.p.) was also tested. Neither dose had a significant effect on temperature (n = 5 per dose, data not shown).

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The bisphosphonates HEBP and AHPrBP but not AHBP inhibit mineral mobilization and lysosomal enzyme release from mouse calvarial bones in tissue culture[1]

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Summary. The effect of 3 bisphosphonates, 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP), 3-amino-1-hydroxy-propylidene-1, 1-bisphosphonate (AHPrBP) and azacycloheptylidene-2, 2-bisphosphonate (AHBP), on the release of minerals (^{40}Ca, ^{45}Ca, P_i) and enzymes from cultured mouse calvaria was investigated in an organ culture system. HEBP and AHPrBP reduced PTH-stimulated mobilization of calcium and inorganic phosphate without affecting the release of lactate dehydrogenase. In contrast, no significant effect by AHBP on mineral mobilization and lysosomal enzyme release could be registered. In parallel with inhibited mineral mobilization, HEBP and AHPrBP inhibited the release of the lysosomal enzyme β-glucuronidase. A possible cellular mechanism of action of bisphosphonates is discussed in the light of these data.
Key words. Mouse; calvarial bone; biphosphonates; mineral mobilization; lysosomal enzymes; calcium release; inorganic phosphate release; tissue culture.

Bisphosphonates are compounds which have profound effects on mineralizing tissues[2-4]. They are chemically characterized by a P-C-P bond which is structurally analogous to the P-O-P bond of inorganic pyrophosphate. In contrast to the naturally occurring pyrophosphate the bisphosphonates are resistent to enzymatic hydrolysis. Three different bisphosphonates have been used in man to treat different disorders in bone and cartilage: (HEBP 'EHDP' 1-hydroxyethylene-1, 1-bisphosphonate), Cl_2MBP (dichloromethylene bisphosphonate) and AHPrBP ('APD' 3-amino-1-hydroxypropylidene-1, 1-bisphosphonate). HEBP inhibits bone resorption and bone mineralization at the same concentrations[5] whereas Cl_2MBP is more potent as an inhibitor of bone resorption than as an inhibitor of bone mineralization[6,7]. Recently it has been reported that AHPrBP also has a preferential inhibitory action on bone resorption at doses where no direct effect on bone mineralization could be registered[8-10]. Thus AHPrBP has been used to prevent bone resorption in diseases such as Paget's disease[8], tumor hypercalcemia[11] and rheumatoid arthritis[12]. We here report some data from a study of the effects of AHPrBP on bone cells in tissue culture, as compared to another bisphosphonate, HEBP. We have also examined the effects of a newly synthesized bisphosphonate, AHBP (azacycloheptylidene-2, 2-bisphosphonate), since this compound has been added to a tooth paste recently marketed in Sweden.

Material and methods. Calvarial bones (frontal and parietal) from 5–7-day-old mice (CsA type) were dissected aseptically, washed in Tyrode's solution and divided along the sagittal suture giving 2 half-calvaria. Care was taken during the dissection procedure not to damage the thin periosteum layer. Calvarial halves from 3–4 litters were pooled and randomized in different groups according to the experimental protocol. Subsequently, the individual half-calvaria were transferred to plastic dishes (A/S Nunc, Copenhagen) containing culture media[13] with and without bisphosphonates and incubated for 48 h at 37 °C in a gas phase of 5% CO_2 in air.

In a 1st type of experiment the bones were prelabelled in vivo by a s.c. injection of 1.5 μCi ^{45}Ca (11 μCi/g, New England Nuclear) 4 days prior to sacrifice. The labelled half-calvaria were cultured separately on grids in plastic dishes containing 5.5 ml of CMRL 1066 medium according to the procedures described by Reynolds[14]. The rate of bone resorption was quantified by following the release of ^{45}Ca (% of initial radioactivity) from the bones to the medium.

In a 2nd type of experiments non-labelled half-calvaria were explanted on grids in multi-well dishes (Linbro Scientific Inc., Hamden, Conn.) containing 2 ml phenol-red free BGJ_b medium.

The magnitude of bone resorption was assessed by following the increase in concentrations in the media of calcium (Ca^{2+}) and inorganic phosphate (P_i). Ca^{2+} was analyzed according to Willis[15] and P_i by the method of Chen et al.[16]. The release of lysosomal enzymes from the explants to the media was assessed by analyzing the activities of β-glucuronidase in media and bone extracts. β-glucuronidase was assayed with phenolphthalein-glucuronidate as substrate[17]. The release of cytosolic enzymes was followed by determining the activities of lactate dehydrogenase (LDH) in media and bones after culture. LDH was assayed by determining the oxidation rate of reduced nicotinamide adenine dinucleotide at 25 °C[18]. Statistical analysis was performed with Student's t-test for unpaired samples.

Synthetic, bovine parathyroid hormone (PTH 1–34) with a potency of 6000 IU/mg was obtained from Beckman, Geneva, Switzerland. CMRL 1066 and BGJ_b medium were from Flow