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Short communication

IN VIVO 'ENKEPHALINASE' INHIBITION BY ACETORPHAN IN HUMAN PLASMA AND CSF

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Thiorphan, the potent inhibitor of 'enkephalinase', has shown some analgesic properties in experimental animals and in man. The possibility that the intravenous infusion of acetorphan, a prodrug of thiorphan (26 μg/kg per min for 60 min), can inhibit plasma and cerebrospinal fluid (CSF) enkephalinase in man in vivo was investigated. A decrease of approximately 65% in enzyme activity was observed in both plasma and CSF. Acetorphan did not induce any significant variation of plasma angiotensin-converting enzyme activity.

Angiotensin-converting enzyme Cerebrospinal fluid 'Enkephalinase' Acetorphan Thiorphan

1. Introduction

Thiorphan is a high affinity inhibitor of 'enkephalinase' (Roques et al., 1980), the enzyme that was found to be identical in man also with a neutral metalloendopeptidase of the kidney (EC 3.4.24.11) (Gafford et al., 1983). It was shown that the drug produces an increase of the striatal [Met^5]enkephalin level (Zhang et al., 1982) and a naloxone-reversible analgesia in mouse (Roques et al., 1980). The esterification of the zinc and arginine binding functions of thiorphan has produced acetorphan, a more lipophilic molecule that easily enters the brain. Acetorphan was 1000 times less potent than thiorphan against purified enkephalinase. However, preincubation of acetorphan with cerebral membranes splits the thioester and ester groups, converting the compound in the more active form, i.e. thiorphan. Therefore, acetorphan could act as a parenterally active inhibitor of enkephalinase (Schwartz et al., 1985). Even if acetorphan, given by intravenous infusion was found to be active in post-myelographic headache (Floras et al., 1983), it is still under investigation whether this enkephalinase inhibitor has an analgesic effect in man and can be considered as a therapeutic agent in pain syndromes. Moreover, little is known about the ability of thiorphan or its prodrug acetorphan to inhibit enkephalinase in man in vivo. In the present study, plasma and cerebrospinal fluid (CSF) enkephalinase activities were measured in humans after intravenous infusion of acetorphan. To evaluate the specificity of acetorphan in inhibiting enkephalinase, the activity of angiotensin-converting enzyme (ACE), which is able to cleave the same enkephalin Gly-Phe amide bond, was also measured in plasma under the same conditions.

2. Materials and methods

2.1. Patients and sample collection

Ten hospitalized patients, requiring lumbar puncture for diagnostic purposes, were included in the study. Their informed consent was obtained after complete explanation about the aim and

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procedure of the study. All patients were subsequently diagnosed as suffering from classic migraine according to the 'Ad Hoc Committee Classification' (1962). All subjects had been drug-free for at least 2 weeks and refrained from eating the night before the study. During the test all patients were in a pain-free period. The procedure began at 8:00 a.m. Lumbar puncture was performed in the lateral decubitis position. The needle was left in position for the duration of the test. The lumbar puncture was atraumatic in all cases. An arm vein blood sample (5 ml) was taken at the same time as each CSF specimen (1 ml). Drug or placebo were given 15 min after the first CSF and blood collection and this was considered time 0. Blood and CSF samples were taken at 30, 60, 120, 180 and 240 min. The patients were divided randomly into two groups, to which either drug or placebo was administered in a single-blind manner. Five subjects (2 females, 3 males, mean age ± S.E.M., 42 ± 6 years) were given an infusion of acetorphan (26 μg/kg per min for 60 min). The 5 other patients (2 females, 3 males, mean age ± S.E.M., 39 ± 7 years) received a saline infusion at the same flow rate as that of acetorphan at time 0. Side-effects were not observed following either drug or saline administration. Blood, collected in heparinized glass tubes, and CSF were centrifuged at 2100 × *g* for 15 min at 4°C. Plasma and CSF were stored at −20°C until assay.

2.2. Measurement of enkephalinase activity

Of each sample 150 μl (plasma diluted 1/30, CSF undiluted) were preincubated for 5 min at 37°C. Incubation, at 37°C for 60 min, was started by adding 50 μl of a solution containing succinyl-alanyl-alanyl-phenylalanyl(7-amido-4-methyl)coumarine, (Suc-Ala-Ala-Phe-AMC) (final concentration 10^{-4} M) dissolved in 50 mM pH 7.4 HEPES/NaOH buffer. Blanks values were obtained in parallel samples by incubating the above-mentioned mixture in the presence of thiorphan (10^{-6} M). The reaction was stopped by the addition of 150 μl thiorphan 10^{-6} M (final concentration) and by heating the samples at 95°C for 15 min. In a second step the incubation medium was further incubated at 56°C for 60 min in the presence of 0.75 μg aminopeptidase M, previously placed at 56°C for 60 min. After heating at 95°C for 15 min the appearance of AMC fluorescence was measured with an Aminco Bowman fluorimeter (exc. 367 nm, em. 440 nm). In each assay the same procedure described above was also applied to the substrate Suc-Ala-Ala-Phe-AMC incubated with 3 μg of thermolysin, this sample to be used as a standard to determine the intensity of fluorescence after complete substrate hydrolysis. The method was a modification of that described by Mumford et al. (1980) and is sensitive enough to detect 50 pg of purified enkephalinase. The inter-assay variation coefficient was 5%. Plasma and CSF enkephalinase had a K_m for the substrate of 58 ± 2 and 54 ± 8 μM and a K_i for [Met^5]enkephalin of 17.1 ± 3.9 and 19.5 ± 3.9 μM respectively. The K_i of plasma and CSF enkephalinase for thiorphan were similar to that for the purified enzyme (our observation) (10^{-9} M). In the assay, each sample was measured in triplicate and the blanks were found to contain approximately 10% of activity.

2.3. Measurement of angiotensin-converting enzyme (ACE) activity

ACE was measured as previously described by Yang and Neff (1972). Briefly, 50 μl of plasma were incubated at 37°C for 30 min with hippuryl-histydyl-leucine (Hip-His-Leu) 10^{-3} M. Blanks were obtained by adding captopril 10^{-6} M to the incubation medium. Fluorescence was recorded with a fluorimeter at exc. 365 nm and em. 495 nm.

2.4. Statistical analysis

Statistical calculations were performed with the analysis of variance for repeated measurements. The values obtained at each time interval after placebo and acetorphan administration were compared further by using the two-tailed Student's t-test for unpaired data.

2.5. Reagents

Substances were obtained as follows: Suc-Ala-Ala-Phe-AMC (Bachem, Switzerland); HEPES,

His-Leu, Hip-His-Leu and thermolysin (Sigma, USA); aminopeptidase M (Pierce Chemical, USA); [Met^5]enkephalin (Peninsula Lab., USA); captopril (Squibb, USA); acetorphan (Laboratoire Biobrojet, France). Purified enkephalinase and thiorphan were gifts of Dr. J.C. Schwartz (Paris, France).

3. Results

3.1. Plasma and CSF enkephalinase

The enzyme activity in plasma (mean ± S.E.M.) before saline and acetorphan infusion, was 88 ± 8.5 and 98.6 ± 3.8 pmol/ml per min respectively. Enkephalinase activity values following acetorphan were significantly reduced at 30 min ($P < 0.05$) and 60 min ($P < 0.01$) as compared to those after saline at the same times. At these time intervals, the enzyme activity after acetorphan was 55% (45.8 ± 6 pmol/ml per min) and 35% (30.4 ± 2.9 pmol/ml per min) of that found after saline (82.8 ± 4.9 and 85.4 ± 5.3 pmol/ml per min respectively). In CSF, the enkephalinase activity (mean ± S.E.M.) was 2.19 ± 0.14 pmol/ml per min before acetorphan and 2.05 ± 0.16 pmol/ml per min before saline. A significant inhibition of enzyme activity following acetorphan infusion was noted at 30 min ($P < 0.05$), 60 min ($P < 0.01$) and 120 min ($P < 0.01$). In these cases, enkephalinase activity was reduced to 52% (0.94 ± 0.19 pmol/ml per min), 37% (0.73 ± 0.11 pmol/ml per min) and 40% (0.79 ± 0.15 pmol/ml per min) of the values obtained after saline (1.79 ± 0.19, 1.95 ± 0.25 and 1.98 ± 0.24 pmol/ml per min) (fig. 1).

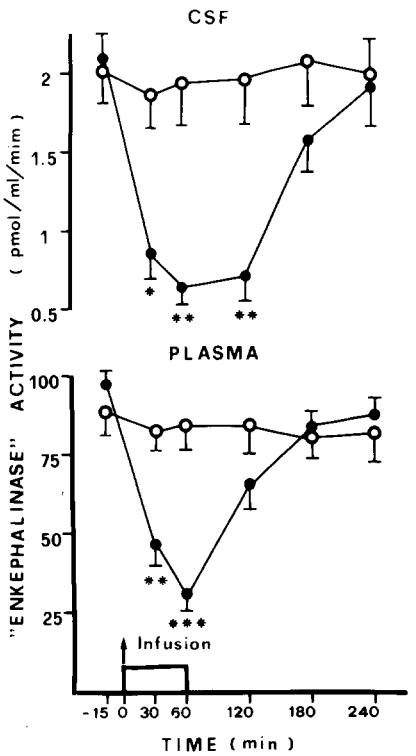

Fig. 1. 'Enkephalinase' activity in plasma and CSF before and after the infusion of acetorphan in 5 subjects (26 μg/kg per min for 60 min) (full circles) and saline in 5 subjects (open circles). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to values obtained following saline infusion.

3.2. Plasma ACE

The pretreatment values (mean ± S.E.M.) for ACE activity in plasma were 10.3 ± 2 and 9.5 ± 2 nmol/ml per min before acetorphan and saline respectively. No significant modification was observed in ACE activity following the infusion of acetorphan when compared to the activity after saline (fig. 2).

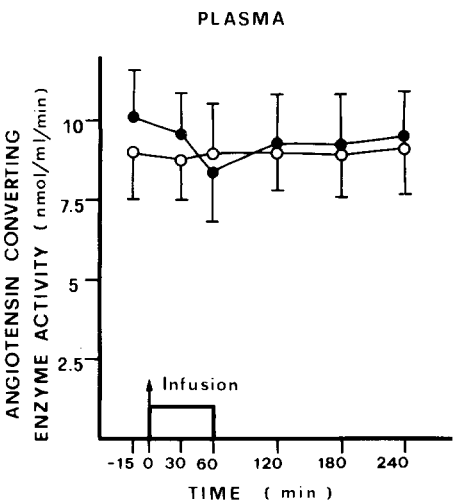

Fig. 2. Plasma angiotensin-converting enzyme activity before and after the infusion of acetorphan in 5 subjects (26 μg/kg per min in 60 min) (full circles) and saline in 5 subjects (open circles).

4. Discussion

Intravenous infusion of acetorphan was found to produce a rapid and strong inhibition of enkephalinase activity in both human plasma and CSF. Since no change of enzyme activity was noted following saline infusion the possibility can be excluded that the lumbar puncture procedure interfered with enzyme activity. The similar inhibition of enkephalinase observed at 30 min in the two body fluids indicates an easy passage of the drug through the blood-brain barrier. Enzyme inhibition lasted for 60 min in plasma and 120 min in CSF and drug infusion was terminated after 60 min. The action of acetorphan was then quickly reversible and this is likely to have depended on the short half-life of the drug in human fluids.

Enkephalinase activity in CSF was approximately 40 times lower than that in plasma and the enzyme present in both fluids showed the same K_i for thiorphan. Following acetorphan administration, the 35% residual activity indicates a proportional and similar inhibition in both CSF and plasma. The elevated enkephalinase inhibition in CSF is consistent with the conversion of acetorphan into the highly potent compound thiorphan by brain membranes (Schwartz et al., 1985). It is likely that human blood and vascular tissue also effect this transformation, thus eliciting the strong inhibition of plasma enkephalinase. Acetorphan did not induce any significant inhibition of ACE. Therefore, at the dosage used, the drug did not act on the activity of ACE, at least in plasma.

As far as the ability of thiorphan to increase the synaptic enkephalin level in animals by inhibiting enkephalinase is concerned (Zhang et al., 1982), the present study shows that this possibility could also be operant in man following the peripheral administration of acetorphan. However, it must be emphasized that our results were obtained in migraine patients. It remains to be found whether the drug produces the same effect in healthy subjects. Furthermore, even in the case of complete enkephalinase inhibition, other enzymes can metabolize enkephalins (Schwartz, 1983) and these enzymes are not necessarily blocked by thiorphan. In addition, despite the fact that enkephalinase inactivates other neuropeptides such as substance P, neurotensin, cholecystokinin, etc. (Turner et al., 1985), inhibition of this enzyme has not been shown to prevent the breakdown of any endogenous neuropeptide other than enkephalins (Schwartz et al., 1985). However, it cannot be excluded that enkephalinase inhibition in the human brain may elicit effects different from those expected only from an increase of enkephalin levels.

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