The Inhibitory Effect of Selank on Enkephalin-Degrading Enzymes as a Possible Mechanism of Its Anxiolytic Activity

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Examination of patients with various forms of anxiety and phobic disorders (according to DSM-4 criteria) demonstrated a considerable shortening of enkephalin half-life and reduced total enkephalinase activity in the blood during generalized anxiety, but not during panic disorders and agoraphobia. This was probably related to low blood concentration of endogenous inhibitors of enkephalin-degrading enzymes in patients with generalized anxiety disorders. Heptapeptide Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro), which attenuates behavioral anxiety reactions and does not cause side effects typical of most anxiolytics, dose-dependently inhibited enzymatic hydrolysis of plasma enkephalin (IC_{50} 15 μM). Selank was more potent than peptidase inhibitors bacitracin and puromycin in inhibiting enkephalinases. These results suggest that high efficiency of Selank in the therapy of anxiety and phobic disorders, including generalized anxiety, is due to its ability to inhibit enkephalin hydrolysis.

Key Words: *anxiety and phobic disorders; enkephalins; enkephalinases; enkephalinase inhibitors; Selank*

Opioid peptides along with other neurochemical systems are involved in the pathogenesis of anxiety and phobic disorders (APD). This is confirmed by high level of anxiety in δ-opioid receptor knockout animals [7] and the dependence of rat brain opioid receptor on behavioral manifestations of anxiety estimated by both radioligand binding assay and polymerase chain reaction [2]. Behavioral tests demonstrated anxiolytic properties of synthetic opioid receptor agonists [3].

Function of endogenous opioid system can be stimulated not only with stable synthetic opioid analogs, but also with inhibitors of enzymes of opioid peptide hydrolysis (*e.g.*, enkephalinase inhibitors).

Here we identified the form of APD characterized by increased activity of enkephalin-degrading enzymes and studied the effects of a new peptide preparation Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro), which attenuates behavioral manifestations of anxiety and does not cause side effects typical of most anxiolytics [1], on enkephalin-degrading enzymes. Selank possesses not only anxiolytic, but also other activities [5] similar to those of opioids [8].

MATERIALS AND METHODS

Blood plasma was obtained from 38 patients with borderline neurotic APD and 15 healthy donors. Enkephalinase activity was estimated by accumulation of radioactive products of ^{3}H-leu-enkephalin enzymatic degradation [6]. The incubation mixture (final volume 50 μl) contained a 10-fold diluted plasma, 10 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl, and 1 μCi ^{3}H-leu-enkephalin (150 nM) with a specific activity of 120 Ci/mmol. Titium label was introduced into the peptide by solid-phase catalytic isotope exchange [4]. The maximum reaction rate (V_{max}) was estimated in the presence of 1.6 mM cold leu-enkephalin. The in-

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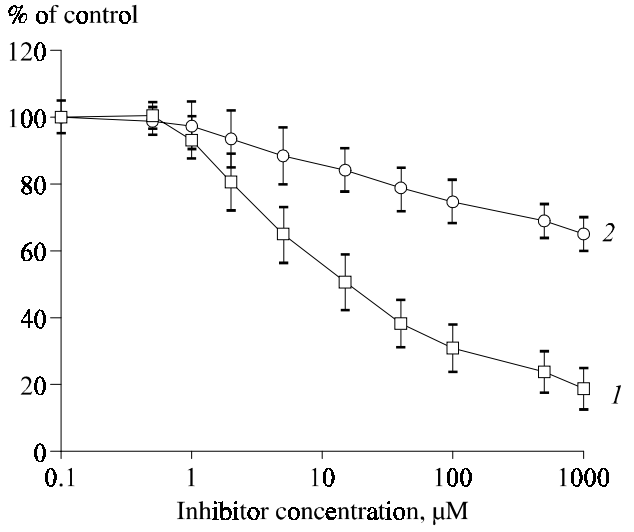

Fig. 1. Inhibitory effects of Selank (*1*) and puromycin (*2*) on enkephalin-degrading enzymes in human plasma. Ordinate: enkephalinase activity.

cubation was carried out at 37°C for 15 min and stooped by adding 5 μl 0.2 M HCl. Products of radioactive leu-enkephalin hydrolysis were separated by thin-layer chromatography on silica gel plates (Merck) in an ethyl acetate-isopropanol-water-acetic acid mixture (40:40:1:19).

Recent studies showed that human plasma contains at least 7 enzymes hydrolyzing enkephalin by various peptide bonds [9]. Under conditions used in our experiments, enkephalin degradation in the polyenzyme system satisfies the criteria for pseudostationary. The rate of this reaction linearly depends on plasma dilution and reaches maximum at a saturating substrate concentration of more than 1 mM [6]. Activity of plasma enkephalin-degrading enzymes was estimated by V_{max} and half-life of leu-enkephalin ($t^1/_2$). The leu-enkephalin half-life was expressed as the reciprocal value of initial reaction rate constant at low substrate concentrations (0.15 μM) comparable to the content of endogenous opioids.

The plasma pooled from 5 healthy donors was used to compare the ability of Selank, puromycin, and bacitracin to inhibit enkephalin-degrading enzymes. The incubation mixture contained 10-fold diluted plasma, 10 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl, 150 nM ^{3}H-leu-enkephalin, and test preparations in concentrations varying from 0.1 nM to 1 mM. The rate of enkephalin hydrolysis in this medium without inhibitors was 5 nmol/min. Each point was the mean of at least 3 independent measurements.

The results were analyzed by Statistica for Windows software.

RESULTS

The mean values characterizing plasma enkephalin-degrading enzyme activity in patients with APD ($t^1/_2$ 2.3±0.5 min and V_{max} 0.16±0.03 μmol/min) did not differ from those in healthy donors ($t^1/_2$=2.0±0.3 min and V_{max}=0.20±0.02 μmol/min).

Enkephalinase activity depended on the form of anxiety disorders classified by DSM-4. Patients with generalized anxiety disorders were characterized by most pronounced changes in the endogenous opioid system manifested in a significant decrease in $t^1/_2$ and V_{max}. These parameters in patients with panic disorders did not differ from normal. In patients with agoraphobia $t^1/_2$ tended to increase compared to the control, which probably reflected behavioral adaptation of the organism under conditions of developing ADP (Table 1).

Parameters V_{max} and $t^1/_2$ characterize the rate of enkephalin degradation by the same enzymes: the higher is the total enzyme activity (V_{max}), the lower is $t^1/_2$. In our experiments, no inverse correlation between these parameters in the total sample or individual groups of patients was found. This can be explained by the presence of endogenous enkephalin-degrading enzyme inhibitors in the blood [9]. Under conditions of reversible inhibition, substrate excess used for estimation of V_{max} partially or completely abolishes the effect of inhibitors on enkephalinase activity and therefore V_{max} reflects true activity of these enzymes, while $t^1/_2$ estimated at low substrate concentration characterizes not only activity of leu-enkephalin-degrading enzymes, but also plasma content of endogenous enkephalinase inhibitors.

The decrease in both parameters in patients with generalized anxiety disorders indicates that short half-life of blood enkephalins is determined by relatively low concentration of endogenous enzyme inhibitors rather than by high content of degrading enzymes.

TABLE 1. Parameters Reflecting Plasma Enkephalinase Activity in Patients with APD (*M*±*m*)

Parameter	Healthy donors (*n*=15)	Generalized anxiety disorders (*n*=13)	Panic disorders (*n*=14)	Agoraphobia (*n*=11)
$t^1/_2$, min	2.3±0.5	1.6±0.03*	2.2±0.6$^+$	3.0±0.8*$^+$
V_{max}, μmol/min	0.20±0.02	0.12±0.03	0.16±0.04	0.18±0.04

Note. p<0.01: *compared to panic disorders; $^+$compared to generalized anxiety disorders.

Taking into account anxiolytic activity of synthetic enkephalin analogues [3], it can be suggested that the therapeutic effect of Selank in patients with generalized anxiety disorders results from deceleration of endogenous enkephalin hydrolysis caused by exogenous enkephalinase inhibitors.

In our experiments, Selank dose-dependently decreased the rate of enkephalin hydrolysis by plasma enzymes (Fig. 1). The concentration of this peptide causing a 50% inhibition of enzyme activity (IC_{50}) was 15 μM. Under these conditions, IC_{50} for peptidase inhibitor bacitracin (59.4 IU/mg, Sigma) is 50 μg/ml (70 μM). Proteinase inhibitor puromycin in all concentrations was also less potent than Selank (Fig. 1).

Thus, the ability of Selank to inhibit enkephalin-degrading enzymes probably underlies the mechanisms of its biological effects. Taking into account activation of the polyenzyme enkephalinase system associated with low content of endogenous enzyme inhibitors in patients with generalized anxiety disorders, it can be hypothesized that the use of Selank for the therapy of APD is pathogenetically determined by its ability to stimulate the opioid system.

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