Effects of Peptide Pro-Gly-Pro-Leu under Conditions of Normal Hemostasis and Thrombus Formation in Rats

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Tetrapeptide Pro-Gly-Pro-Leu *in vitro* effectively inhibited platelet aggregation over the entire range of studied concentrations (10^{-12}-10^{-3} M). In concentrations of 10^{-9}-10^{-3} M it exhibits fibrinolytic activity and in concentrations of 10^{-5}-10^{-3} M has anticoagulant properties. Under *in vivo* conditions the analyzed tetrapeptide in a dose of 1 mg/kg increased anticoagulant, total and fibrin depolymerizating activities and increased activity of plasminogen activator. Intravenous administration produced more pronounced anticoagulant effect and leads to a greater increase in activity of plasminogen activator than intranasal administration. Tetrapeptide Pro-Gly-Pro-Leu also exerts antithrombotic effect. Preliminary repeated intranasal administration of the peptide before blood clot formation reduces the weight of fresh fibrin clots.

Key Words: *tetrapeptide Pro-Gly-Pro-Leu; blood clotting; fibrinolysis; anticoagulant activity; thrombosis*

Many disorders associated with abnormal hemostasis lead to the development of atherosclerosis, thromboembolism, diabetes, and other diseases. Pathological thrombogenesis plays a very important role in these processes. Prevention and treatment of thrombosis are essential for clinical practice. Currently existing antithrombotic drugs are not effective enough. Therefore, the development of highly efficient drugs with a wide range of activities and minimum side effects is an urgent problem [3]. These substances were found among regulatory peptides, including products of hydrolysis of structural protein collagen (glyprolines). *In vitro* and in *vivo* experiments [1,2] demonstrated anticoagulant, antiplatelet, fibrin-depolynerizing, and fubrinolytic effects of glyprolines. It is well known that diabetes is accompanied by depression of the anticoagulation system of the body. Some glyprolines (Pro-Gly-Pro, Pro-Gly, Phe-Pro-Gly-Pro) exhibit protective anti-diabetogenic effects simultaneously stabilizing the hemostatic parameters. There is evidence that amino acid leucine produces a normoglycemic effect [9], but no information on the effect of leucine on blood clotting is available. We hypothesized that addition of leucine to glyproline Pro-Gly-Pro can yield a substance positively affecting the functions of the anticoagulation system.

Here we studied anticoagulant and antiplatelet effects of tetrapeptide Pro-Gly-Pro-Leu.

MATERIALS AND METHODS

Tetrapeptide Pro-Gly-Pro-Leu synthesized in the Institute of Molecular Genetics, Russian Academy of Sciences; leucine (Leu) domestically produced; commercial preparations of fibrinogen and thrombin (Pharmaceuticals Plant in Kaunas) were used. Blood clotting was analyzed using APTT-test diagnostic kit (Tekhnologiya-Standart Ltd.). ADP (AppliChem GmbH) was used to determine platelet aggregation. The work was carried out on 75 albino random-bred male rats weighing 200-250 g.

The test preparations were administered into *v. jugularis* or intranasally. Control animals received 0.85% NaCl under the same conditions. The blood

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was taken from *v. jugularis* 10 min and 1 h after drug administration and stabilized with 3.8% sodium citrate (9:1 blood:citrate ratio). For evaluation of hemostasis, we measured activated partial thromboplastin time (APTT) [6], total (TFA) and nonenzymatic fibrinolytic activity (NFA) on non-stabilized fibrin plates [7], activity of tissue plasminogen activator (TPA) on standard fibrin plates [3], and platelet aggregation by the method [4] on Agregometr device (Moscow State University production) using ADP at a final concentration of 15 μM as aggregation inductor.

Antithrombotic effects of the tetrapeptide and leucine were studied on the model of thrombosis by the method of Wessler in our modification. The animals intranasally received tetrapeptide in a dose of 1 mg/kg body weight (group 1), leucine in a dose equivalent to its content in the peptide (0.33 mg/kg, group 2), or 0.85% NaCl solution (group 3, control) for 3 days. One hour after the third dose, blood clot formation against the background aminazine treatment (2.5%, 0.5 ml/200 g) was induced. After 30-45 min, *v. jugularis* was dissected and the fascia and ligaments were removed. The venous segment was isolated with clamps at a distance of 0.7-1 cm from each other. Thrombin solution (0.1 ml; 6-7 NIH/kg body weight) was injected with a syringe via a fine needle into the isolated vein segment full of blood. After 10-15 min, the clamps were removed. Operaton field was covered with a cotton swab soaked in 0.85% NaCl solution. The animals were left for 60 min for possible lysis of newly-formed fibrin clot. Then blood was taken from the jugular vein (2 ml) for measurements of biochemical parameters, and the sections of vessels with experimentally induced clots were made. Fibrin clots were removed from the sections, washed with saline, dried at room temperature for 1 h, and weighed. Antithrombotic effect of the test substances administered to animals before the start of thrombosis was evaluated by the weight of the clots.

The results were processed using Student's *t*-test.

RESULTS

The test tetrapeptide exhibited moderate anticoagulant activity. In concentrations of 10^{-5}-10^{-3} M, it significantly lengthened APTT by 10-20% in comparison with the control. The tetrapeptide added to the plasma from healthy animals in concentrations of 10^{-10}-10^{-3} M significantly increased TFA by 1.5-2.9 times in comparison with the control. In addition, it demonstrated fibrin-depolymerizing activities in concentrations of 10^{-9}-10^{-3} M and significantly increasing NFA by 1.52-3.74 times in comparison with the control. Hence, Pro-Gly-Pro-Leu is characterized by non-enzymatic fibrinolytic effects on fibrin non-stablilized by factor XIIIa. These results indicate that the tetrapeptide can weaken hydrogen bonds and hydrophobic interactions between the molecules of fibrin monomers and provide its transition to monomeric forms.

Pro-Gly-Pro-Leu produced a significant inhibition of platelet aggregation (by 33.4%) upon incubation with the samples of platelet-rich plasma followed by ADP stimulation of the mixture in the wide range of drug concentrations (10^{-11}-10^{-3} M).

Amino acid leucine in concentrations equivalent to its content in the peptide had no effect on hemostasis parameters (APTT, TFA, NFA, and platelet aggregation).

Thus, Pro-Gly-Pro-Leu *in vitro* exerted antithrombotic, anticoagulant and fibrin-depolymerizing activities.

Single intravenous administration tetrapeptide *in vivo* in a dose of 1 mg/kg increased plasma anticoagulant activity by 28% (APTT test) 10 min postinjection. Administration of leucine insignificantly reduced the time of clot formation in comparison with the control. Under these conditions, administration of the tetrapeptide insignificantly inhibited platelet aggregation and significantly increased the parameters of the fibrinolytic system of rat blood plasma: TFA by 43.5%, NFA by 32%, and TPA by 153% (Table 1). Thus, under these conditions the tetrapeptide enhanced

TABLE 1. Parameters of the Hemostasis System in Rat Plasma 10 min after Single Intravenous Injection of Tetrapeptide Pro-Gly-Pro-Leu (1 mg/kg) and Leucine (0.33 mg/kg) (%; *M*±*m*)

Parameter	NaCl (control; *n*=7)	Pro-Gly-Pro-Leu (*n*=8)	Leucine (*n*=5)
APTT	100.0±8.1	128.0±8.3*	91.7±9.9
TFA	100.0±4.2	143.5±6.9**	128.8±15.9
NFA	100.0±2.8	131.7±8.9*	121.2±6.8
TPA	100.0±7.6	253.1±23.1**	73.7±20.3
PA	100.0±11.7	69.0±8.3	117.2±1.4

Note. Here and in Tables. 2 and 3: PA: platelet aggregation. *p<0.05, **p<0.01 in comparison with the control.

TABLE 2. Parameters of the Hemostasis System in Rat Plasma 1 h after Repeated (3 days) Intranasal Administration of Tetrapeptide Pro-Gly-Pro-Leu (1 mg/kg) and Leucine (0.33 mg/kg) (%; *M*±*m*)

Parameter	NaCl (control; *n*=9)	Pro-Gly-Pro-Leu (*n*=9)	Leucine (*n*=9)
APTT	100.0±2.2	118.0±2.6**	95.2±1.6
TFA	100.0±2.6	145.2±19.8*	102.7±6.8
NFA	100.0±5.2	139.1±5.6**	105.4±4.4
TPA	100.0±6.6	164.4±12.9**	99.7±27.8
PA	100.0±10.0	82.5±7.0	93.8±9.6

TABLE 3. Parameters of the Hemostasis System in Rat Plasma and Weight of Clots under conditions of Blood Clot Formation after 3-Fold Intranasal Administration of Tetrapeptide Pro-Gly-Pro-Leu (1 mg/kg) and Amino Acid Leu (0.33 mg/kg) (%; *M*±*m*)

Parameter	NaCl (control; *n*=7)	Pro-Gly-Pro-Leu (*n*=8)	Leucine (*n*=8)
APTT	100.0±5.7	117.4±5.5*	98.3±5.1
TFA	100.0±8.5	156.4±15.5**	88.3±5.9
NFA	100.0±5.3	169.2±18.6**	94.1±14.5
TPA	100.0±7.9	134.70±6.16**	98.2±7.4
PA	100.0±6.0	93.9±14.7	98.1±12.5
Weight of clots, %	100.0±3.6	31.5±6.5**	70.4±15.8

the anticoagulant and fibrinolytic activities of rat blood plasma and at the same time had no significant effect on platelet aggregation. Leucine administered via the same route had no effect on APTT, TFA, NFA, and platelet aggregation.

After intranasal administration of the test preparations, the following results were obtained. One hour after the third dose of Pro-Gly-Pro-Leu, plasma anticoagulant activity increased by 18% (by APTT test) and TFA, NFA and TPA increased by 45, 39, and 64%, respectively, in comparison with the control (Table 2). After intranasal administration, tetrapeptide is absorbed by the nasal and nasopharyngeal mucosa and then enters the blood. On the other hand, it is known that glyprolines quickly penetrate into the subcortical and cortical structures through lymphatic vessels within the perineural space [8]. Some effects of intranasal administration result from peptide influences on peripheral reflexogenic zones.

Intranasal administration of leucine had no significant effect on anticoagulant, fibrinolytic, and antithrombotic characteristics of rat plasma (Table 2).

Thus, repeated intranasal administration of Pro-Gly-Pro-Leu moderately increased anticoagulant activity and enhanced fibrin-depolymerizing blood plasma activity and TPA.

It should be noted that intravenous administration exerted a greater anticoagulant effect and increase in TPA, while TFA and NFA, parameters of rat blood plasma fibrinolytic activity, were comparable after intravenous and intranasal administration.

Later we studied antithrombotic effects of the tetrapeptide. Under conditions of experimental thrombosis against the background of preliminary administration of tetrapeptide, the weight of fresh thrombi in the vessel significantly decreased. At the same time, anticoagulant and fibrinolytic activities of blood plasma in group 1 animals (peptide administration) significantly increased. In these animals, APTT increased by 17.4% in comparison with the control, TFA, NFA, and TPA increased by 56.4, 69.2, and 34.7%, respectively compared with the control (Table 3).

Thus, repeated intranasal administration of Pro-Gly-Pro-Leu prevented thrombogenesis, *i.e.* the peptide exhibited antithrombotic activities.

Under these experimental conditions, leucine had no antithrombotic action.

As for the mechanism of Pro-Gly-Pro-Leu action, the fact that anticoagulant and fibrinolytic activities of rat blood plasma increased in all administration routes indicates that this effect is due to both direct and indirect mechanisms. Thee presence of glyprolin receptors on the endothelium has not been proved yet, but the existence of specific Pro-Gly-Pro binding sites on cytoplasmic membrane of the basal nuclei in rat brain recently have been reported [5]. Taking into account the

fact that Pro-Gly-Pro-Leu administered via the intranasal route crosses the blood-brain barrier and enters the brain structures, we can hypothesize that this tetrapeptide exerts its effects through specific receptors.

Individual amino acids constituting Pro-Gly-Pro-Leu peptide do not affect the parameters of blood coagulation in animals [7]. Only this combination of amino acids in the molecule determines anticoagulant and fibrinolytic activity of Pro-Gly-Pro-Leu.

Thus, our data attest to possible participation of Pro-Gly-Pro-Leu tetrapeptide in the regulation of the anticoagulation systems and drive us to a conclusion that glyproline Pro-Gly-Pro-Leu can be used as a promising antithrombotic drug.

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