CHANGES IN THE NOCICEPTIVE RESPONSE TO THERMAL STIMULATION IN RATS FOLLOWING ADMINISTRATION OF N-TERMINAL ANALOGS OF THE ADRENOCORTICOTROPIC HORMONE

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Melanocortins (MCs) are an increasingly studied class of regulatory peptides exerting a wide range of biological effects. All naturally occurring MCs share a His-Phe-Arg-Trp fragment (HFRW) corresponding to the sequence of amino acid residues 6–9 of the adrenocorticotropic hormone ($ACTH_{6-9}$), which is also a central active component of ACTH. Attaching the Pro-Gly-Pro (PGP) sequence to the C-end of the peptide extends the duration of the peptide's effect. The aim of this study was to investigate the effects of $ACTH_{6-9}$-PGP (HFRWPGP) on the spinal and supraspinal mechanisms involved in the nociceptive response in rats and to compare them to those of its structural analog $ACTH_{4-7}$-PGP (MEHFPGP). $ACTH_{6-9}$-PGP effects were studied following the intraperitoneal administration of the peptide at doses 0.5, 1.5, 5, 15, 50, 150, or 450 μg/kg 15 minutes before the hot plate and tail flick tests. $ACTH_{4-7}$-PGP effects were studied under the same conditions at the following doses: 50, 150 and 450 μg/kg. We found that $ACTH_{6-9}$-PGP administered intraperitoneally at 5 or 150 μg/kg induced a pronounced reduction in pain sensitivity 15 and 45 minutes after the injection ($p = 0.04$); this effect was implemented via supraspinal mechanisms. In the tail flick test, 150 μg/kg $ACTH_{6-9}$-PGP increased pain sensitivity, with the participation of segmental spinal mechanisms ($p = 0.04$). $ACTH_{4-7}$-PGP did not have any effect on the studied mechanisms of pain sensitivity. Thus, unlike $ACTH_{4-7}$-PGP, $ACTH_{6-9}$-PGP can both increase pain sensitivity and exert an analgesic effect.

Keywords: regulatory peptide, ACTH, pain, hot plate, tail flick

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Compliance with ethical standards: the study was approved by the Ethics Committee of Kursk State Medical University (Protocol № 3 dated October 27, 2015). The animals were treated in strict compliance with the Declaration of Helsinki, Directive 2010/63/EU of the European Parliament and the Council (September 22, 2010) on the protection of animals used for scientific purposes, and Good Laboratory Practice guidelines established by the Order 708n of the Ministry of Healthcare of the Russian Federation (August 23, 2010).

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ИЗМЕНЕНИЕ ТЕМПЕРАТУРНОЙ БОЛЕВОЙ ЧУВСТВИТЕЛЬНОСТИ У КРЫС ПОСЛЕ ВВЕДЕНИЯ N-КОНЦЕВЫХ АНАЛОГОВ АДРЕНОКОРТИКОТРОПНОГО ГОРМОНА

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Меланокортины (МК) — один из активно исследуемых классов пептидных регуляторов с широким спектром биологических эффектов. В структуре всех природных МК присутствует общий фрагмент — His-Phe-Arg-Trp (HFRW), соответствующий последовательности с 6-го по 9-й аминокислотный остаток молекулы адренокортикотропного гормона ($АКТГ_{6-9}$) и являющийся ее активным центром. Показано, что присоединение к С-концу аминокислотной последовательности Pro-Gly-Pro (PGP) приводит к пролонгации действия пептидов. Целью работы было изучить влияние эффектов $АКТГ_{6-9}$-PGP (HFRWPGP) на спинальные и супраспинальные механизмы формирования болевой чувствительности у крыс, а также сравнить их с эффектами его структурного аналога — $АКТГ_{4-7}$-PGP (MEHFPGP). Эффекты $АКТГ_{6-9}$-PGP были исследованы при его внутрибрюшинном введении в дозах 0,5, 1,5, 5, 15, 50, 150 и 450 мкг/кг за 15 мин до начала опыта по изучению температурной болевой чувствительности у крыс с использованием тестов «горячая пластина» и «отдергивание хвоста». Эффекты $АКТГ_{4-7}$-PGP были исследованы в аналогичных условиях в дозах 50; 150 и 450 мкг/кг. Показано, что $АКТГ_{6-9}$-PGP в дозах 5 и 150 мкг/кг вызывал выраженное снижение температурной болевой чувствительности через 15 и 45 мин после его внутрибрюшинного введения ($p = 0,04$), реализованного на супраспинальном уровне. В тесте «отдергивание хвоста» $АКТГ_{6-9}$-PGP в дозе 150 мкг/кг повышал температурную болевую чувствительность с участием сегментарных спинальных механизмов ($p = 0,04$). $АКТГ_{4-7}$-PGP не оказывал влияния на исследованные механизмы болевой чувствительности. Таким образом, установлено, что $АКТГ_{6-9}$-PGP, в отличие от $АКТГ_{4-7}$-PGP, способен обладать как анальгетическими, так алгическими эффектами.

Ключевые слова: регуляторный пептид, АКТГ, боль, горячая пластина, отдергивание хвоста

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Regulatory melanocortin peptides and their fragments exert a wide range of biological effects. This phenomenon has inspired the creation of synthetic melanocortin analogs for structure-function analysis and therapeutic applications [1, 2]. It is known that N-terminal regions of the adrenocorticotropic hormone (ACTH) have a neurotropic effect and affect the body's response to pain [3]. The His-Phe-Arg-Trp sequence found at $ACTH_{6-9}$ is required for the activation of all melanocortin receptor types [4]. Some of $ACTH_{6-9}$ effects are retained after the Pro-Gly-Pro (PGP) tripeptide is attached to its C-terminus so as to enhance resistance to carboxypeptidases [5]. However, the impact of the $ACTH_{6-9}$-PGP peptide on sensitivity to pain has not been studied so far. At the same time, a synthetic fragment $ACTH_{4-7}$-PGP, which is structurally close to $ACTH_{6-9}$-PGP and is also an active ingredient of a pharmaceutical drug Semax, has similar effects [3]. Therefore, it is important to investigate the neurotropic action of $ACTH_{6-9}$-PGP as part of the structure-function analysis of N-terminal ACTH fragments.

The aim of this work was to study the effect of the $ACTH_{6-9}$-PGP peptide on the spinal and supraspinal mechanisms of nociceptive response in rats and to compare them to the effects of $ACTH_{4-7}$-PGP.

METHODS

Our experiments were carried out in 121 male Wistar rats weighing 150–190 g. The animals were kept in cages (10 rats per cage) in standard housing conditions at a 12/12 light/dark cycle at controlled ambient temperature (22 ± 2 °C). The rats were fed a standard pellet diet and had free access to water. The experiments were carried out between 9 am and 15 pm.

Ten experimental and one control groups were formed consisting of 11 rats each. Each experimental group received a single dose of either $ACTH_{6-9}$-PGP or $ACTH_{4-7}$-PGP (Semax) from the dose range specified below. The peptides used in the experiment had been synthesized in advance at the Institute of Molecular Genetics (RAS). $ACTH_{6-9}$-PGP was dissolved in 0.9% normal saline. Fifteen minutes before the experiment, 7 experimental groups received a single intraperitoneal injection of $ACTH_{6-9}$-PGP at one of the following doses: 0.5, 1.5, 5, 15, 50, 150, or 450 μg/kg. $ACTH_{4-7}$-PGP was also dissolved in normal saline and administered intraperitoneally to the animals from the 3 remaining experimental groups at a single dose of 50, 150 or 450 μg/kg 15 min before the tests. The control group received equivalent amounts of normal saline (1 ml per 1 kg body weight).

Pain response to thermal stimulation was assessed using the hot plate and the tail flick tests [6] and the corresponding equipment: a hot plate (model LE7406) and a tail-flick meter (model LE7106) (PanLab Harvard Apparatus; Spain). The hot plate test consisted of 4 trials separated by 15-min breaks and conducted at 53 °C. In this test, the nociceptive threshold was measured once before the injection and 3 times after the injection. Each animal was placed on a hot plate and the latency to the first behavioral response (hind paw licking, jumping) to the nociceptive stimulus was measured. In the tail flick test, the middle section of the tail was exposed to the heat stimulus of fixed intensity (set to 50 units) and the latency to tail flicking was recorded. In this test, 5 measurements were taken at 15-min intervals: 2 pretreatment measurements of the baseline nociceptive threshold (averaged) and 3 measurements of the nociceptive threshold after the injection. The maximum possible effect (MPE) was calculated using the following formula: [6]:

$$MPE = \frac{LP_{ost} - LP_{re}}{CO_{time} - LP_{re}} \times 100\%,$$

where LP_{ost} is posttreatment latency, LP_{re} is pretreatment latency, CO_{time} is cut-off time (45 s in the hot plate test and 9 s in the tail flick test).

Statistical analysis was carried out in MS Excel 2016 (Microsoft; USA), Statistica 13.3 (StatSoft; USA) and the R environment (The R Foundation for Statistical Computing; Austria). Normality of data distribution was assessed using the Shapiro Wilk test. The equality of variances was tested using Levene's test (lawstat). The obtained results were expressed as a median (Me), lower (25) and upper (75) quartiles (Q1 and Q3). Statistical significance was assessed using the nonparametric univariate Kruskal–Wallis test; post-hoc intergroup comparisons were done using the Mann–Whitney U test and the Benjamini–Hochberg procedure. The results were considered significant at $p < 0.05$

RESULTS

A single injection of the $ACTH_{6-9}$-PGP peptide produced a dose-dependent effect on the MPE value in the hot plate test (see Table). The analgesic effect was the most pronounced for the 5 μg/kg dose 15 and 45 min after the injection, with a significant 2.5-fold ($p = 0.04$) and 7-fold ($p = 0.02$) elevation of the nociceptive threshold, respectively. At a lower dose of 1.5 μg/kg, the peptide did not have any considerable impact on pain sensitivity. However, further dose reduction to 0.5 μg/kg changed the trend: 30 min after the $ACTH_{6-9}$-PGP injection, the MPE value dropped by 193% ($p = 0.15$) and remained at this low level for at least 45 min ($p = 0.07$).

Increasing the $ACTH_{6-9}$-PGP dose to 15 μg/kg did not have any significant effect. However, the peptide tended to increase sensitivity to pain 45 min after the injection when administered at 50 μg/kg ($p = 0.1$). Further dose increase to 150 μg/kg was accompanied by a significant elevation of the nociceptive threshold 15 min ($p = 0.04$) and 45 min ($p = 0.04$) after the injection. When administered at 450 μg/kg, $ACTH_{6-9}$-PGP did not have any significant effect on the studied parameters.

No statistically significant differences in the studied parameters were observed between the rats in the experimental groups following the injection of $ACTH_{4-7}$-PGP at all studied doses in comparison with the controls.

Based on the results of the tail flick test, no reliable changes in the nociceptive threshold were observed in the rats injected with 0.5, 1.5, 15, 50, and 450 μg/kg of $ACTH_{6-9}$-PGP (see Table).

Although no significant effect of $ACTH_{6-9}$-PGP administered at 5 and 150 μg/kg was observed in the hot plate test 30 min after the injection, the peptide did affect the rats' sensitivity to heat in the tail flick test 30 min after its administration: at 150 μg/kg the nociceptive threshold was significantly lower ($p = 0.04$) and at 5 μg/kg the peptide tended to increase pain sensitivity ($p = 0.1$).

The tail flick test did not reveal any significant changes in pain sensitivity in the animals treated with $ACTH_{4-7}$-PGP.

DISCUSSION

Biological effects of melanocortin peptides are implemented via a variety of melanocortin receptors (MCRs). Specifically, MCR1 is expressed in the neurons of the periaqueductal gray, MCR3 is expressed in the cortex and the thalamus, whereas MCR4, in the brain stem. The activation of these brain structures plays an important role in nociception by reducing pain progression [7, 8]. Considering that supraspinal structures are involved in the nociceptive response to thermal stimulation in the hot plate test [6], the interaction between $ACTH_{6-9}$-PGP and

Table 1. Effects of $ACTH_{6-9}$-PGP and $ACTH_{4-7}$-PGP on pain sensitivity in rats in the hot plate and tail flick tests, MPE (Me (Q1; Q3))

MPE, % / Group	15 min	30 min	45 min	15 min	30 min	45 min
	Hot plate test			Tail flick test		
Control	12.92 (–6.05; 26.83)	1.9 (–6.93; 5.84)	3.58 (–3.18; 9.53)	15.53 (–22.91; 29.78)	15.69 (–5.07; 28.69)	–17.81 (–46.16; 25.12)
$ACTH_{6-9}$-PGP 0.5 μg/kg (*n* = 11)	4.41 (–16.20; 7.73)	–18.11 (–38.62; –0.72)	–18.73 (–31.94; –7.92)	17.08 (6.06; 50.00)	0.05 (–30.59; 23.43)	–14.31 (–41.03; 5.79)
$ACTH_{6-9}$-PGP 1.5 μg/kg (*n* = 11)	8.98 (–0.59; 19.74)	16.28 (8.43; 23.68)	–9.05 (–26.00; 11.33)	11.68 (8.08; 38.79)	22.42 (–3.37; 54.11)	–5.95 (–7.98; 19.13)
$ACTH_{6-9}$-PGP 5 μg/kg (*n* = 11)	45.44* (23.88; 54.69)	28.05 (–1.46; 42.62)	31.11* (27.57; 36.58)	–7.73 (–28.83; 16.98)	–8.76 (–19.43; 12.29)	8.6 (–8.97; 41.44)
$ACTH_{6-9}$-PGP 15 μg/kg (*n* = 11)	16.48 (11.57; 48.20)	11.75 (–0.76; 29.79)	10.83 (–11.48; 46.64)	0.62 (–8.11; 22.87)	–6.11 (–17.38; 12.50)	1.96 (–3.77; 22.24)
$ACTH_{6-9}$-PGP 50 μg/kg (*n* = 11)	3.57 (–9.45; 23.48)	–4.09 (–32.97; 7.96)	–10.42 (–23.20; 2.20)	11.65 (–13.77; 40.37)	19.97 (9.45; 40.40)	15.73 (–5.16; 20.34)
$ACTH_{6-9}$-PGP 150 μg/kg (*n* = 11)	41.02* (16.37; 66.96)	10.06 (2.88; 29.73)	30.40* (19.13; 37.31)	–18.04 (–44.05; 6.37)	–31.67* (–36.45;–21.85)	–20.85 (–43.43; 8.58)
$ACTH_{6-9}$-PGP 450 μg/kg (*n* = 11)	24.15 (9.36; 26.39)	6.61 (–7.00; 17.15)	4.56 (–2.13; 6.92)	3.77 (–21.28; 23.96)	11.16 (–28.31; 40.84)	–5.78 (–22.03; 38.22)
$ACTH_{4-7}$-PGP 50 μg/kg (*n* = 11)	0.45 (–14.1; 37.27)	11.01 (–18.57; 23.17)	10.23 (–0.03; 42.15)	1.21 (–22.86; 38.97)	22.7 (–58.53; 32.46)	–4.63 (–47.24; 25.36)
$ACTH_{4-7}$-PGP 150 μg/kg (*n* = 11)	12.07 (–5.06; 34.59)	12.48 (0.64; 31.16)	1.52 (–5.75; 16.44)	10 (–43.69; 26.44)	0.1 (–44.11; 27.93)	–3.91 (–40.36; 21.57)
$ACTH_{4-7}$-PGP 450 μg/kg (*n* = 11)	20.37 (8.63; 27.69)	12.19 (–9.18; 32.12)	21.9 (–9.19; 35.83)	25.78 (1.54; 80.88)	–16.71 (–35.19; 25.67)	11.83 (–27.97; 26.38)

Note: * — differences are significant ($p \leq 0,05$) in comparison with the control group at the same time points (according to the Mann-Whitney U test and the Benjamini–Hochberg procedure).

melanocortin receptors localized in the brain can affect pain sensitivity.

MCR4 is found in the spinal cord [3, 8]; a $ACTH_{6-9}$-PGP-induced change in their activity can affect the function of segmental mechanisms involved in nociception, which was observed in our flick tail test. It is still unclear how melanocortin receptors mediate the effect of ACTH analogs; it is hypothesized that there is at least one previously undescribed receptor subtype involved [9, 10].

The observed differences in the intensity and directionality of the effects induced by different peptide doses can be explained by the interaction of the peptide with various types of MCRs it encounters when travelling across the brain or by some aspects of intracellular mechanisms of its action. Specifically, transmembrane and subsequent intracellular signaling from an MCR can be implemented via different pathways depending on the concentration of the ligand: through cAMP activation, by inositol phosphates [1, 11], Ca^{2+} and protein kinases [1]. This is true for all types of melanocortin receptors [1]. The direction, intensity and duration of the nociceptive response to a stimulus are determined by the type of the activated signal transduction mechanism [1, 2]. These facts are consistent with our findings demonstrating the opposite dose-dependent trends in responding to the thermal stimulus following $ACTH_{6-9}$-PGP administration; these effects are typical of regulatory peptides as a separate class of bioactive substances [11, 12].

The opposite trends in the nociceptive response following $ACTH_{6-9}$-PGP administration observed in our study are also consistent with the literature reports on the effects of ACTH fragments and analogs on nociception [3, 13]. It could be hypothesized that the mechanisms of the $ACTH_{6-9}$-PGP effect on the response to pain are similar to those of its structural analog $ACTH_{4-7}$-PGP (Semax), which reduces sensitivity to pain and exerts its effects via opioid and serotonin receptors [3].

Because the $ACTH_{6-9}$-PGP sequence is required for the activation of all types of melanocortin receptors [4], the effects of $ACTH_{6-9}$-PGP on pain sensitivity (compared to those of $ACTH_{4-7}$-PGP) observed in our study may result from the interaction of the peptide with all types of MCRs in CNS structures involved in nociception and antinociception. Interestingly, we did not observe the pain-reducing effect of $ACTH_{4-7}$-PGP described by other authors [3]. Considering that the character of pain response can be largely determined by the features of a specific animal breed or line [14, 15], the differences observed in our study may be to some extent explained by the use of Wistar rats vs outbred rodents exploited in the studies cited above.

CONCLUSIONS

In the hot plate test, a reduction in pain sensitivity was observed 15 and 45 minutes after the intraperitoneal injection of 5 and 150 μg/kg $ACTH_{6-9}$-PGP; this reduction was mediated by supraspinal mechanisms. Other studied doses of the peptide did not produce any effect on the nociceptive response. In the tail flick test, the injection of 150 μg/kg $ACTH_{6-9}$-PGP increased pain threshold, with the participation of segmental spinal mechanisms. No effect on pain response was observed for $ACTH_{4-7}$-PGP. Our findings broaden the knowledge of physiological effects of N-terminal ACTH analogs and can provide a theoretical rationale for developing neurotropic pharmaceutical agents based on these compounds.

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