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Involvement of endogenous nitric oxide in the effects of kyotorphin and its synthetic analogue on immobilization and cold stress-induced analgesia

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The aim of the present study was to investigate the involvement of endogenous nitric oxide (NO) in the effects of neuropeptide kyotorphin (Kyo) and its synthetic analogue D-kyotorphin (D-Kyo) on immobilization and cold stress-induced analgesia (SIA). In scientific literature, Kyo is considered as neuromodulator. D-Kyo is more stable enzimatically. Some studies demonstrate that Kyo is a possible substrate for neuronal and inducible NO synthase, also the NO system is stress-limiting and plays an important role in initiation and maintenance of pain.

Kyo and D-kyo were synthesized by the Group of Antimetabolites at the Institute of Molecular Biology, Bulgarian Academy of Sciences. The proposed synthetic route was based on well-established liquid-phase methods of peptide synthesis – active esters, using *tert*-butyloxycarbonyl (Boc) group for protection of the Nα-amino group according to the protocol previously described in the literature. Male *Wistar* rats were used in acute immobilization and cold stress models. The evaluation of nociceptive effects was carried out using the paw pressure (PP) and hot plate (HP) tests. Kyo and D-Kyo (both in dose 5 mg/kg), L-NAME (10 mg/kg), and L-Arginine (1 mg/kg) were dissolved in saline and were injected intraperitoneally.

The result showed that endogenous NO is differently involved in the effects of peptides, which may be due to different implication of opioid and non-opioid components of SIA. Probably, L- or D-form of the peptide is also important, as well as the type of the stressor and its neurochemical signature.

Key words: kyotorphin, D-kyotorphin, nitric oxide, stress-induced analgesia.

INTRODUCTION

Kyotorphin (Kyo) is a dipeptide (L-Tyr-L-Arg) (Fig. 1A) synthesized in specific brain regions [1]. The highest levels were found in the lower brain stem and dorsal spinal cord, areas closely associated with the pain regulatory system [2, 3]. D-Kyotorphin (D-Kyo or L-Tyr-D-Arg) (Fig. 1B) is a synthetic analogue of Kyo. Both peptides bind to a specific Kyo-receptor and induced Met-enkephalin release at rates of approximately 4 times basal release [4]. Literature data showed that Kyo-receptor is identified in the membrane-preparations of the brain, which suggests that it plays a physiological significance in the neurotransmission as a neurotransmitter/neuromodulator [5]. However, D-Kyo shows enhanced analgesic activity, i.e. 5.6-fold higher than that observed with Kyo. Takagi and co-workers [1] suggested that this effect is a result of protease resistance conferred by the substitution of L-arginine (L-Arg) with a D-arginine residue [6].

It is also known that acute and chronic stress induces biochemical changes affecting both pain threshold and behaviour [7]. Stressors such as immobilization and cold exposure can cause stress-induced analgesia (SIA) [8]. It can be resolved into an opioid, when it is antagonized by naloxone, but also a non-opioid component [9].

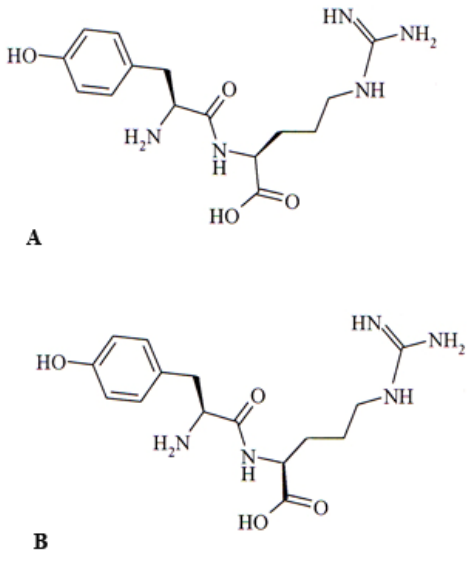

Fig. 1. Structural formula of: A. kyotorphin (L-Tyr-L-Arg); B. D-kyotorphin (L-Tyr-D-Arg).

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Rat experiments have demonstrated that the nitric oxide (NO) system fulfils the main criteria of a stress-limiting system [10]. Earlier it was reported that the mechanism of NO-induced antinociception involved opioid components and was also dependent on brain NO [11]. NO is an unique neurotransmitter was synthesized by the enzyme nitric oxide synthase (NOS) and plays an important role in initiation and maintenance of pain [12]. Also, it is known that Kyo, as well as L-Arg, are possible substrates for neuronal and inducible NOS [13, 14]. The aim of the present study was to investigate the involvement of endogenous NO in the effects of kyotorphin and D-kyotorphin on immobilization and cold stress-induced analgesia.

EXPERIMENTAL

Chemistry. All reagents and solvents were of reagent grade and used without further purification. Amino acid derivative Boc-Tyr-OH was prepared according to the general procedure of Pozdnev [15] and Boc-Tyr-OSu – according to [16].

Active ester procedure (AE). Nα-protected tyrosine succinimide ester (1 mmol) was dissolved in l ml dimethylformamide (DMF), and 1.2 mmol arginine (L- or D- form) in 1 ml DMF was added to it. The pH was adjusted to 8–9 with 5% $NaHCO_3$, and the mixture was stirred at room temperature overnight. After reduced pressure evaporation, the residue was partitioned between 0.5 M $NaHSO_4$ and *n*-BuOH. The acidic aqueous layer was extracted twice with *n*-BuOH (2 × 30 ml) and the combined organic extracts were washed with 10% $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and evaporated *in vaccuo* to a smaller volume;

Trifluoroacetic acid (TFA) deprotection. The fully protected peptides were treated with TFA (1/10 molar ratio) in the presence of anisole for 30 min at room temperature to remove the Boc-protecting groups. The crude peptides were purified by suitable crystallization. The pure TFA salt obtained in this way was converted to the target product by treatment with ion-exchange resin Amberlite IR-45, dissolved in water and lyophilized.

Analyses. The purity of the peptides and structural identity was established by TLC, analytical HPLC and electrospray mass spectometry. TLC was carried out on silicagel 6OF254 pre-coated (Merck) aluminum plates, with the use of the following solvent systems: A = *n*-butanol:acetic acid:water (4:1:5); B = chloroform:methanol:water (80:30:5). Visualization was done with either UV, or ninhydrin. For the analytical gradient RP-HPLC, a Merck-Hitachi liquid chromatograph, was used. A water/0.1% TFA buffer, pH 2.25, and a linear acetonitrile gradient were used for elution on a 100-5 Nucleosil Ci8 column. To check the purity of compounds, a 5–100% acetonitrile gradient in 30 min was performed. Optical rotation was measured with a Perkin-Elmer Model 141 polarimeter.

Animals. Male *Wistar* rats (180–200 g) were used. The animals were housed in groups of 6 per cage and kept under a normal 12 h light/dark cycle and 22 ± 2°C temperature. Rats had free access to food and water.

Acute models of stress: Immobilization stress (IS). The animals were placed for 1 hour in a plastic tube with adjustable plaster tape on the outside so that the animals were unable to move. There were holes for breathing.

Cold stress (CS). The animals were placed in a refrigerating chamber at 4°C for 1 hour.

Nociceptive tests. The evaluation of antinociceptive effects was carried out using the paw pressure (PP) test of Randall and Selitto [17] and hot plate (HP) test.

Paw-pressure test. The changes in the mechanical nociceptive threshold of the rats were measured by using an analgesimeter (Ugo Basile). The pressure was applied to the hind-paw and the pressure (*g*) required to elicited nociceptive responses such as squeak and struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Hot plate test. The latency of response to pain was measured from the moment of placing an animal on a metal plate (heated to 55 ± 0.5ºC) to the first signs of pain (paw licking, jumping). The cut-off time was 30.

Drugs and treatment. Kyotorphin and D-kyotorphin (both in dose 5 mg/kg) were synthesized by the Group of Antimetabolites at the Institute of Molecular Biology, Bulgarian Academy of Sciences. NOS inhibitor L-N^G-nitroarginine ester (L-NAME) (10 mg/kg) and L-Arginine (L-Arg, 1 mg/kg) were obtained from Sigma. All drugs were dissolved in sterile saline (0.9% NaCl) solution and were injected intraperitoneally (i.p.). The control group was not submitted to stress procedure and was injected with saline 1 ml/kg, i.p. The nociceptive tests were performed 15 min after peptide injection.

The experiments were approved by the Animal Care and Use Committee of the Medical University, Sofia.

Data analysis. The results were statistically assessed by analysis of variance (ANOVA) followed by Dunnetts's multiple comparison test. Values are mean ± S.E.M. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

The proposed synthetic route was based on well-established liquid-phase methods of peptide synthesis – active esters, using *tert*-butyloxycarbonyl (Boc) group for protection of the Nα-amino group (Fig. 2) according to the protocol previously described in literature [18]. The strategy of the minimal side-chain protection was adopted. Thus, the phenolic group of tyrosine and δ-guanidino group of arginine were unprotected.

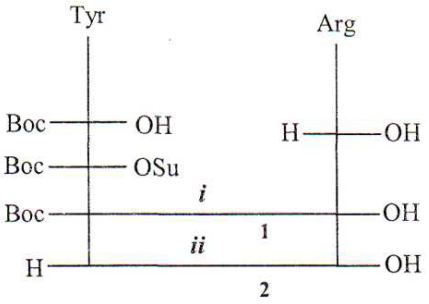

Fig. 2. Scheme for the synthesis of kyothorphin and D-kyotorphin. *i)* 5% $NaHCO_3$, DMF; *ii)* 3 N HCl/EtOAc (TFA).

The Kyo and D-Kyo were synthesized by condensation of the preliminary prepared Boc-Tyr-OSu with L-Arg or D-Arg, respectively. The reaction carried out for 16–24 hours at room temperature resulted in high yields.

After isolation and purification, the peptides were identified and characterized by optical rotation, TLC, analytical HPLC, mass-spectra and elemental analysis.

The investigations started 15 min after intraperitoneal injection of each of the peptides. Our results showed that in PP test Kyo ($p < 0.01$) and D-Kyo ($p < 0.01$) (both at a dose of 5 mg/kg, i.p.) administered immediately after stress procedure significantly inhibited 1 hour immobilization stress-induced analgesia (ISIA) at the beginning of the experiment. On the 30th min only Kyo had kept this effect, while D-Kyo significantly potentated pain threshold ($p < 0.05$) (Fig. 3).

L-NAME (10 mg/kg, i.p.) and L-Arg (1 mg/kg, i.p.) were injected 20 min before investigated peptides and their interactions have been studied after each stress model. Co-administration of Kyo and D-Kyo with L-NAME or L-Arg significantly decreased analgesia induced by IS and CS and measured by PP and HP tests (Figs. 3, 4, 5 and 6).

In PP test L-NAME or L-Arg significantly decreased inhibiting effect of Kyo on ISIA. Their effect on D-Kyo was the same only on the 15th min, while on the 30th min they significantly decreased its increasing pain threshold (Fig. 3).

Injected immediately after 1 hour CS, Kyo ($p < 0.01$) significantly inhibited cold stress-induced analgesia (CSIA) during the whole investigated period, while D-Kyo injected after 1 hour CS procedure decreased significantly the analgesic effect of CS on the 45th min ($p < 0.01$) from the beginning of the experiment (Fig. 4).

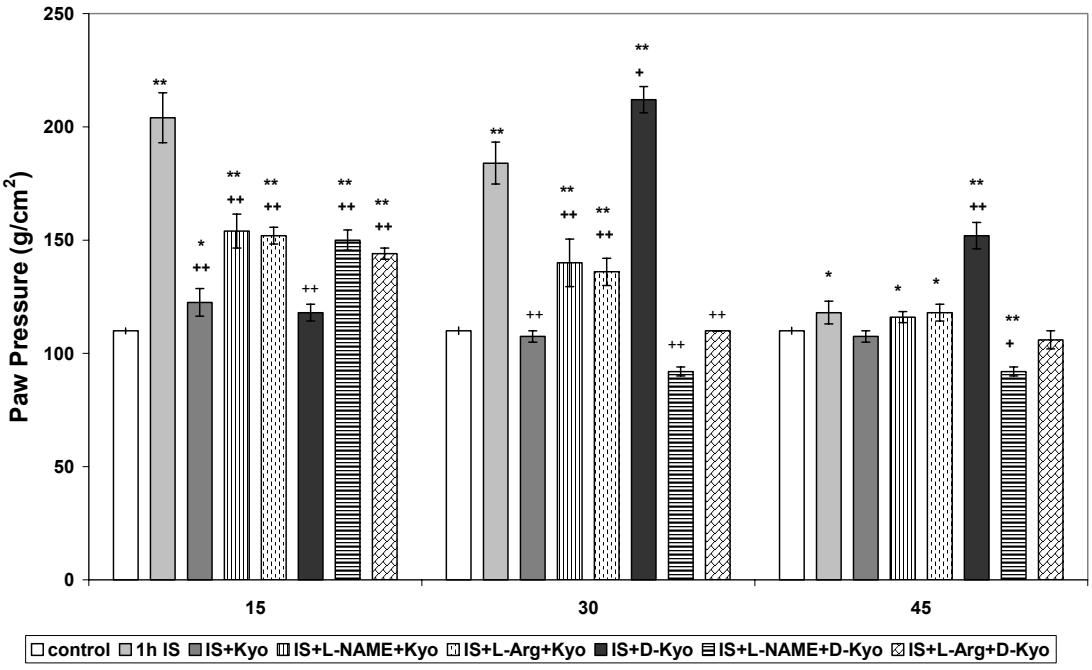

Fig. 3. Effects of Kyo and D-Kyo (both in 5 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) and L-Arg (1 mg/kg, i.p.) on nociception measured with paw pressure test after 1 hour immobilisation stress (IS) in male *Wistar* rats ($n = 5$). Mean values ± S.E.M. are presented. *$p < 0.05$, **$p < 0.01$ vs. control; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. IS.

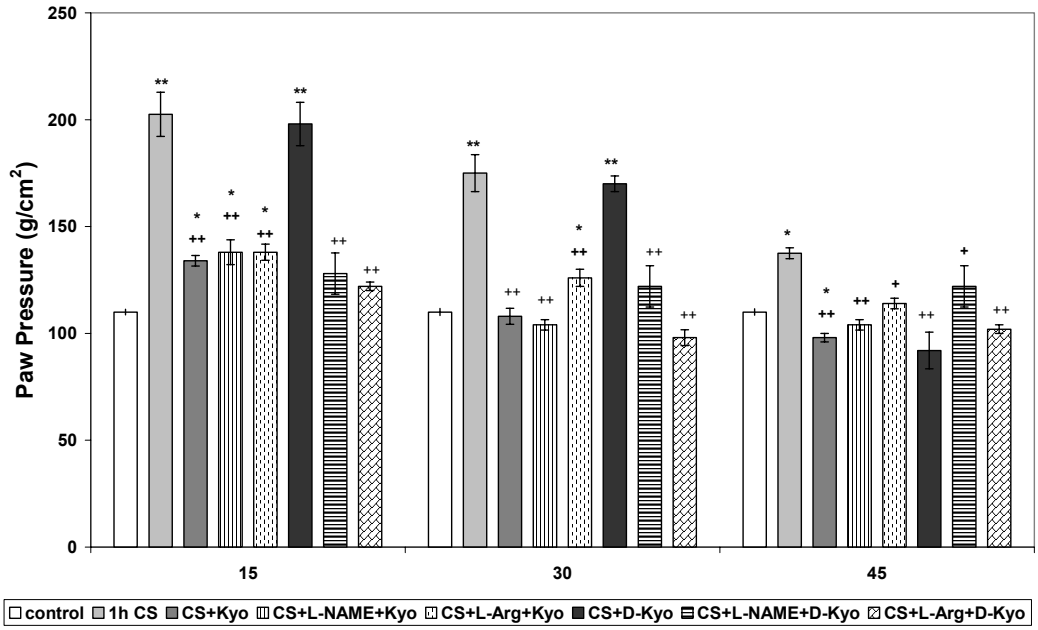

Fig. 4. Effects of Kyo and D-Kyo (both in 5 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) and L-Arg (1 mg/kg, i.p.) on nociception measured with paw pressure test after 1 hour cold stress (CS) in male *Wistar* rats ($n = 5$). Mean values ± S.E.M. are presented. *$p < 0.05$, **$p < 0.01$ vs. control; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. CS.

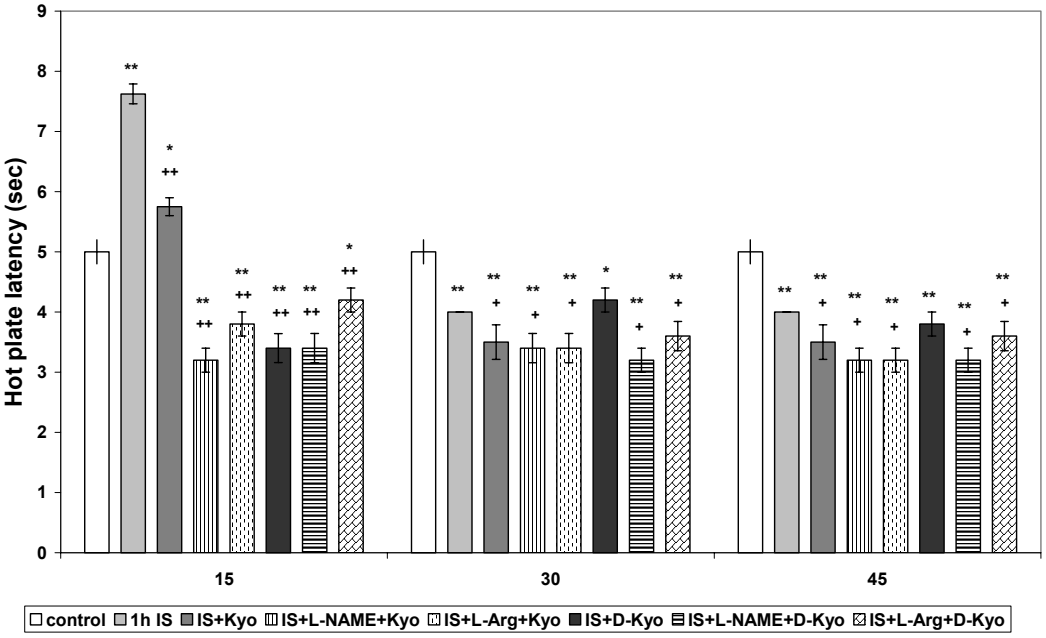

Fig. 5. Effects of Kyo and D-Kyo (both in 5 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) and L-Arg (1 mg/kg, i.p.) on nociception measured with hot plate test after 1 hour immobilisation stress (IS) in male *Wistar* rats (n = 5). Mean values ± S.E.M. are presented. *$p < 0.05$, **$p < 0.01$ vs. control; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. IS.

L-NAME and L-Arg significantly decreased inhibiting effect of D-Kyo on CSIA on the 45th min. Co-administrations L-NAME+Kyo and L-Arg+Kyo showed pain thresholds commensurable to that of Kyo administered after CS (Fig. 5).

In HP test IS significantly increased HP latency only on the 15th min compared to control group ($p < 0.01$). This enlargement on analgesic activity was significantly decreased by Kyo ($p < 0.01$) and D-Kyo ($p < 0.01$) (Fig. 5).

In Fig. 6 CS increased HP latency on the 15th min ($p < 0.01$) and 30th min ($p < 0.05$) compared to control. Kyo significantly inhibited CSIA ($p < 0.05$). D-Kyo showed the same effect, where HP latency was more pronounced on the 30th min ($p < 0.01$) and was shorter than control.

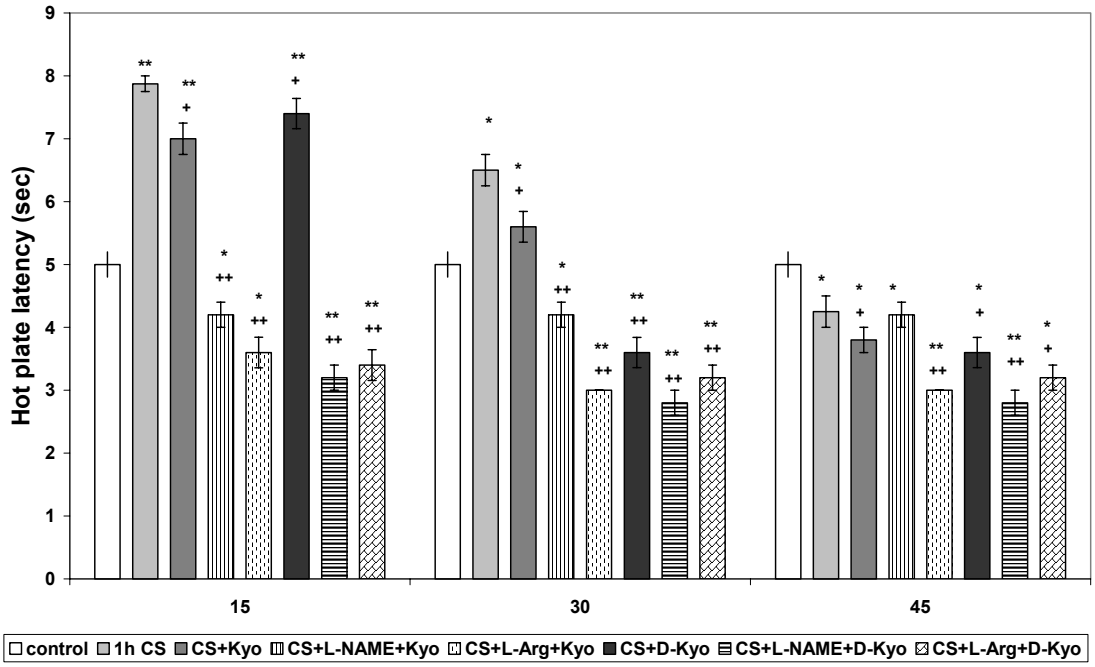

Fig. 6. Effects of Kyo and D-Kyo (both in 5 mg/kg, i.p.) and their combination with L-NAME (10 mg/kg, i.p.) and L-Arg (1 mg/kg, i.p.) on nociception measured with hot plate test after 1 hour cold stress (CS) in male *Wistar* rats ($n = 5$). Mean values ± S.E.M. are presented. *$p < 0.05$, **$p < 0.01$ vs. control; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. CS.

Co-administration of Kyo and D-Kyo with L-NAME or L-Arg significantly increased inhibiting effects of peptides on ISIA and CSIA measured by HP test (Figs. 5 and 6).

NO is an exclusively important and many-sided regulator of a number of physiological functions in animals. It also acts as a neurotransmitter itself and/or as a neuromodulator, influences plastic properties of the neurons, in particular the phenomenon of long-lasting potentiation. NO production occurs in the spinal cord after activation of NMDA receptors and gives rise to acute pain [19].

It is known that stress activates the hypothalamic-pituitary-adrenal (HPA) axis by stimulating neuronal activity within the paraventricular nucleus of the hypothalamus [20]. Some workers have reported that HPA axis responses to neural stimuli, which are not dependent on immune factors, can be modulated by NO and also NO plays an important role in regulating the response of the HPA axis to various stresses. Data in literature showed that endogenous NO influenced nociceptive effects induced by IS and thermogenesis [21].

Our previous data showed that Kyo and D-Kyo applied alone exerted well marked and time dependent analgesic effects, reduced by naloxone. Also, they modulated ISIA and CSIA [22, 23]. Injection of L-NAME or L-Arg before Kyo or D-Kyo showed different effects on ISIA and CSIA in two nociceptive tests. These findings indicate that endogenous NO is differently involved in the effects of peptides, which may be due to different implication of opioid and non-opioid components in two types of SIA – immobilization and cold.

Our unpublished observation and literature data showed that the non-opioid system is mostly involved in cold stress, while both systems – opioid and non-opioid are equally presented in immobilization stress [8]. On the other hand, Kyo, as well as L-Arg, are possible substrates for neuronal and inducible NOS [13, 14]. Literature data revealed that endogenous opioid peptides and NO mediated a wide variety of physiological processes including pain transmission and SIA [7]. The morphological studies present evidence for the existence of a signaling pathway between an opioidergic and the NO systems in the hypothalamus of the rat brain [24]. Also, L- or D-form of the peptide is important for possible NO production.

In conclusion, we suggest that there is a different kind of involvement of endogenous nitric oxide in the mechanisms of nociception of Kyo and D-Kyo after immobilization and cold stress, which may be due to different implication of opioid and non-opioid components of SIA. Probably L- or D-form of the peptide is also important, as well as type of the stressor and its neurochemical signature.

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УЧАСТИЕ НА ЕНДОГЕННИЯ АЗОТЕН ОКСИД В ЕФЕКТИТЕ НА КИОТОРФИН И НЕГОВ СИНТЕТИЧЕН АНАЛОГ ВЪРХУ ИМОБИЛИЗАЦИОННА И СТУДОВА СТРЕС-ИНДУЦИРАНА АНАЛГЕЗИЯ

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(Резюме)

Целта на настоящата работа е да се изследва участието на ендогенния азотен оксид (NO) в ефектите на невропептида киоторфин (Kyo) и неговия синтетичен аналог D-киоторфин (D-Kyo) върху имобилизационна и студова стрес-индуцирана аналгезия (SIA). В научната литература Kyo е известен като невромодулатор, а D-Kyo е по-стабилен на ензимно разграждане. Някои изследвания показват, че Kyo е възможен субстрат за невронална и индуцибилна NO синтаза, а NO система е стрес-лимитираща и играе важна роля в инициирането и поддържането на болката.

Синтезата на Kyo и D-Kyo бе извършена в института по молекулярна биология при БАН, и бе основана на добре известната методика за пептиден синтез в разтвор – метод на активираните естери, при който се използва третична-бутилоксикарбонилна (Boc) група за защита на Nα-амино групата. За острите модели на имобилизационен и студов стрес бяха използвани мъжки плъхове линия *Wistar*. Оценяването на ноцицептивните ефекти беше извършвано с paw pressure (PP) и hot plate (HP) тестове. Kyo и D-Kyo (5 mg/kg), L-NAME (10 mg/kg), и L-аргинин (1 mg/kg) бяха разтворени във физиологичен разтвор и инжектирани интраперитонеално.

Резултатите показаха, че ендогенният NO по различен начин участва в ефектите на пептидите, което може би е свързано с различното участие на опиоидната и неопиоидна компонента на SIA. Вероятно L- и D-формата на пептидите е също важна, както типа стресор и неговият неврохимичен „подпис“.