# Effects of Kyotorphin and D-Kyotorphin on Pain Perception and Blood ACTH and Corticosterone Concentrations after Heat Stress in Rats

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**Abstract:** *Aims & Objectives: The present study consists in evaluation of the effects of the dipeptides Kyotorphin (Kyo) and D-Kyotorphin (D-Kyo) on nociception and plasma levels of adrenocorticotrophic hormone and corticosterone after 1 hour of heat stress in rats. Materials & Methods: Kyo and D-Kyo`s effects on nociception were evaluated by Paw pressure and Hot plate test after 1 hour of heat stress. Plasma levels of both hormones were determined by Radio immunological assays. Conclusions: Since a decrease in heat-stress-induced analgesia and ACTH and CORT plasma levels was observed we assumed that both dipeptides take part in the body`s anti-stress system.*

**Keywords:** Kyotorphin, D-Kyotorphin, Heat stress, Nociception, Anti-stress system

## 1. Introduction

Activation of the hypothalamus-pituitary-adrenal (HPA) axis represents the main neuroendocrine mechanism during stress resulting in acute increase of circulating adrenocorticotrophic hormone (ACTH) and glucocorticoids plasma levels, extremely important for the proper adaptation to different types of stress [9]. Thus ACTH and glucocorticoids are among the indicators of stress [4, 25, 40].

The dipeptide Kyotorphin (L-Tyr-L-Arg, Kyo) was first described by Takagi et al. [42] and isolated initially from bovine brain extracts. Additionally it was isolated also from mouse, rat, guinea pig, rabbit, squirrel, and human brain [16, 33, 52]. Kyo is released by the nerve terminals after depolarization and binds to specific receptors [51]. It`s known that Kyo activates cortical neurons directly, while indirectly influences μ- and δ-opioid receptors, exerting naloxone-reversible and prolonged analgesia due to release of met-enkephalin (Met-Enk) and β-endorphin [23, 42, 43]. D-Kyotorphin (D-Kyo, L-Tyr-D-Arg) was synthesized in 1980 [55]. It represents an optical isomer of Kyo with stronger analgesic effect due to indirect effects on μ- and δ-opioid receptors. Similarly to Kyo D-Kyo induces naloxone-reversible and prolonged analgesia due to Met-Enk and β-endorphin release [34, 35, 43, 55]. Analgesia induced after stress is a common phenomenon in many animal species, and could be triggered by different stressors – immobilization, low or high temperature, social stress [18, 20, 28, 32, 45]. Immobilization, cold and heat stresses increase antinociception in tail-flick, hot-plate and formalin tests [1, 2, 3, 14]. Decrease in pain perception is regarded as stress-induced analgesia (SIA), and two components, an opioid and a non-opioid one take part in its mechanisms of development [17, 19, 20, 21]. The opioid one is naloxone- or naltrexone-reversible [27]. After heat stress the opioid component of SIA-development is the better expressed, while after cold stress it`s the non-opioid one to be the better

expressed; after immobilization stress both the components are equally expressed [6,36].

**The aim** of the present study was to evaluate the effects of Kyo and D-Kyo on analgesia after heat stress, along with ACTH and CORT plasma levels.

## 2. Materials and Methods

### 2.1 Animals

All the experiments were carried out on male Wistar rats (180-200 g). Animals were housed in groups of 8 per cage and kept under a normal 12 h light/dark cycle and $22 \pm 2°C$ temperature, with free access to food and water. All the experiments were conducted between 9.00 and 12.00 a.m., and according to the ''Principles of laboratory animal care'' 9 (NIH publication №85-23, revised 1985), and the rules of the the Animal Care and Use Committee of the Medical University of Sofia.

### 2.2 Drugs and treatment

The peptides were dissolved in 0.9%-saline and intraperitoneally (i.p.) administered. The dose/response curve pointed out a dose of 5 mg/kg for both Kyo and D-Kyo administered after the end of heat stress. The control group received 1ml/kg of saline. Evaluation of nociception began immediately after termination of stress or 15 min after peptides administration.

### 2.3 Nociceptive Tests:

#### 2.3.1 Mechanical nociceptive stimulus method - *Paw pressure (PP) test*
The changes in the mechanical nociceptive threshold of the rats were measured by analgesimeter (Ugo Basile). Pressure was applied to the hind-paw and the pressure (*g*) required to elicit nociceptive response (squeak and struggle) was taken

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as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw [38].

### 2.3.2 Thermal nociceptive stimulus method - Hot plate (HP) test:

The latency of response to pain stimulus was measured from the moment of placing the animal on a metal plate (heated to $55 \pm 0.5°C$) till the first signs of pain (paw licking, jumping). The cutoff time in order to avoid paw damage was 30 sec.

## 2.4 Stress Model

**1 hour of heat stress (1h HS)** was provoked by placing the animals for 1 hour in thermal chamber ($38\pm1°C$) and 45-50% of humidity.

## 2.5 Radio Immunological Assays

After heat stress the animals were injected with Kyo or D-Kyo (at 5 mg/kg, i.p.). Part of them was decapitated by guillotine, without anesthesia, 15 min after peptides` injection. Another part of the animals were decapitated on the 30th min, and the last one - on the 45th min after peptides` administration. Blood was collected in heparinized syringes put on ice, and centrifuged at $4°C$ at 10000 rpm for 10 min. Plasma obtained was collected by eppendorf pipette and was kept at minus 20 $°C$ till the evaluations of ACTH and CORT concentrations were performed. Radioimmunoassay (RIA) of the plasma levels of ACTH and CORT was performed. ACTH plasma levels were evaluated by BRAHMS ACTH RIA, the values represented in pg/ml. CORT plasma levels were evaluated by RIA kit (CIS, Bio Intenational, Paris), the values represented in nmol/l.

## 2.6 Statistical Analysis

The results were statistically assessed by one-way analysis of variance ANOVA followed by t-test comparison. Values are mean $\pm$ S.E.M. Values of $p\leq 0.05$ were considered to indicate statistical significance.

# 3. Results

Kyo increased pain threshold on the 15th ($p<0.001$) and 30th ($p<0.01$) min of the experiment, while D-Kyo increased pain during the whole investigated period ($p<0.001$) compared to the control animals (Figure 1).
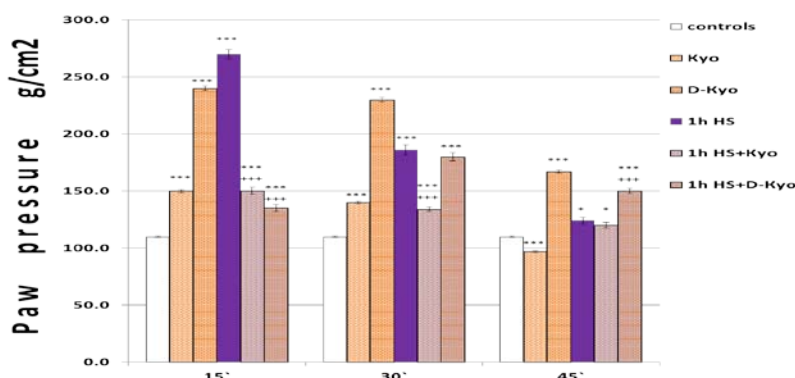


**Figure 1**. Effects of Kyo and D-Kyo (both at a dose 5 mg/kg, i.p.) on pain threshold after one hour of heat stress (1h HS) evaluated by PP- test. Data are represented as mean values ± S.E.M. Pain thresholds of animals with Kyo and D-Kyo, animals after 1h HS, and animals after 1h HS+Kyo and D-Kyo were compared to controls (***$p<0.001$, *$p<0.05$);1h HS+Kyo and 1h HS+D-Kyo were compared to 1h HS ($^{+++}p<0.001$).

Kyo prolonged HP-latency during the whole investigated period, while D-Kyo prolonged HP-latency on the 15th ($p<0.01$) and the 30th ($p<0.05$) min of the evaluated time compared to the controls (Figure 2).
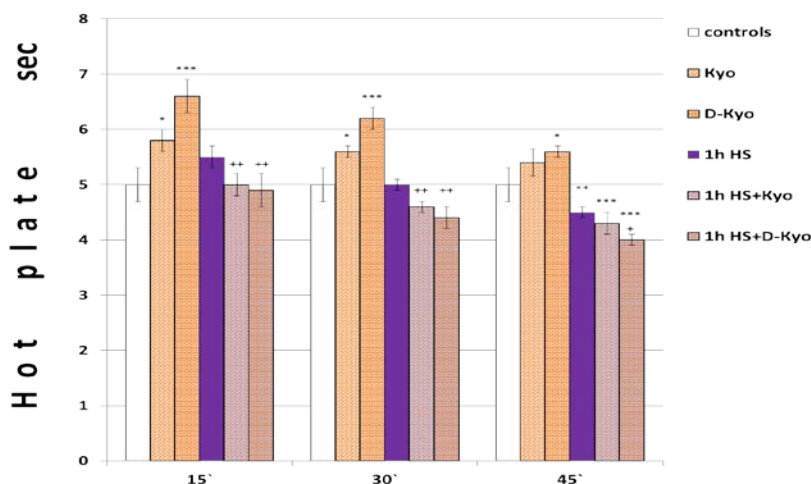
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**Figure 2.** Effects of Kyo and D-Kyo (both at a dose 5 mg/kg, i.p.) on pain threshold after one hour of heat stress (1h HS) evaluated by HP-test. Data are represented as mean values ± S.E.M. Pain thresholds of animals with Kyo and D-Kyo, animals after 1hHS, and animals after 1h HS+Kyo and D-Kyo were compared to controls (***$p<0.001$, **$p<0.01$, *$p<0.05$); 1h HS+Kyo and 1h HS+D-Kyo were compared to 1h HS ($^{++}p<0.01$, $^{+}p<0.05$).

After 1h HS Kyo led to a statistically relevant decrease in pain threshold on the 15th and the 30th min ($p<0.001$), and also to a statistically relevant shortage ($p<0.01$) of HP-latency of the animals for the same time of investigation compared to animals after 1h HS without the peptide. Pain threshold on the 45th min and HP-latency on the 15th min were comparable to the control values (Figure 1 and 2). After 1h HS D-Kyo decreased the pain threshold on the 15th min ($p<0.001$) of the experiment compared to animals after 1h HS without the peptide. On the 30th min the pain threshold was comparable to 1h HS, while on the 45th min a statistically relevant increase compared to 1h HS was observed (Figure 1). D-Kyo shortened ($p<0.01$) HP-latency during the whole investigated period compared with animals after 1h HS. On the 15th min the HP-latency was comparable to the controls (Figure 2). In a second series of experiments ACTH and CORT plasma levels were evaluated after 1h HS and the peptides. 1h HS increased in a statistically relevant degree ($p<0.001$) both plasma levels of ACTH and CORT compared to controls during the whole investigated time. The highest levels observed were on the 15th min (Figure 3 and 4).
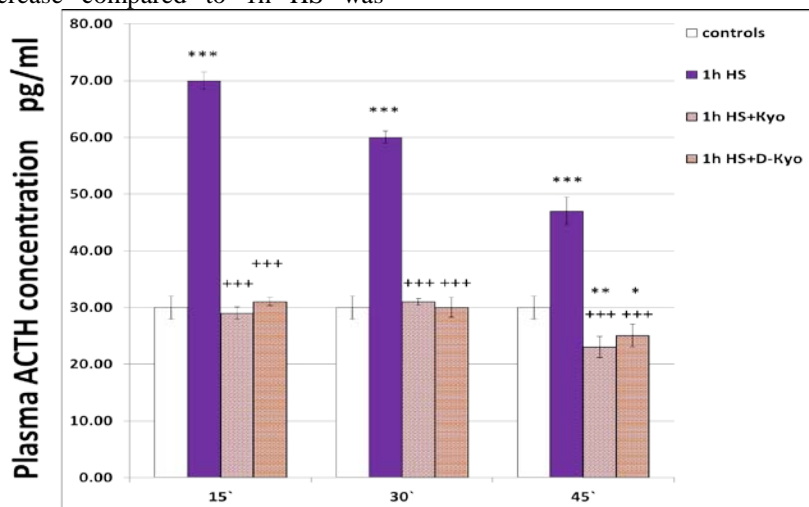


**Figure 3:** Effects of Kyo and D-Kyo on ACTH-plasma levels (pg/ml) after one hour of heat stress (1h HS). Data are represented as mean values ± S.E.M. 1h HS-,1h HS+Kyo- and 1h HS+D-Kyo- ACTH-plasma levels were compared to controls (***$p<0.001$; **$p<0.01$; *$p<0.05$,); 1h HS+Kyo- and 1h+D-Kyo- ACTH- plasma levels were compared to 1h HS-ACTH-plasma levels ($^{+++}p<0.001$; $^{+}p<0.05$).
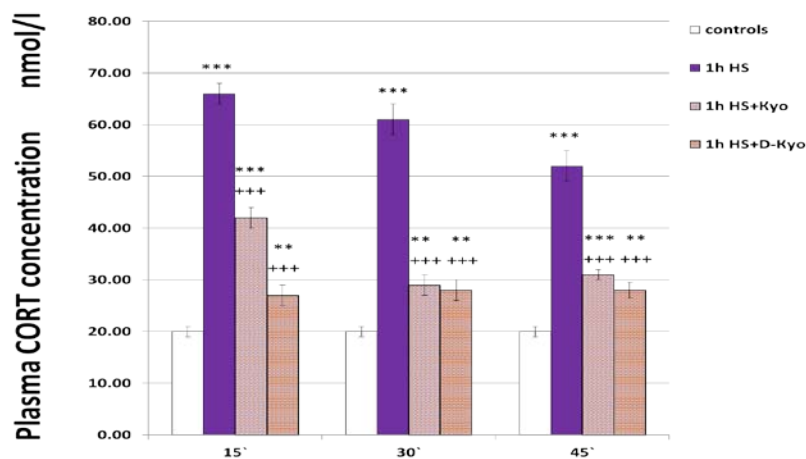


**Figure 4:** Effects of Kyo and D-Kyo on CORT-plasma levels (nmol/L) after one hour of heat stress (1h HS). Data are represented as mean values ± S.E.M. 1h HS-,1h HS+Kyo- and 1h HS+D-Kyo- CORT-plasma levels were compared to controls ( (***$p<0.001$;**$p<0.01$;*$p<0.05$,);1h HS+Kyo-and 1h HS+D-Kyo-CORT-plasma levels were compared to 1h HS-CORT-plasma levels ($^{+++}p<0.001$;$^{+}p<0.05$).

Both Kyo ($p<0.001$) and D-Kyo ($p<0.001$) administered after 1hHS decreased ACTH and CORT plasma levels compared to the controls and 1h HS (Figure 3 and 4). After 1h HS Kyo led to a stronger decrease ($p<0.01$) in ACTH plasma level compared D-Kyo on the 15th and 45th min of the experiment (Figure 3). After 1h HS D-Kyo led to a stronger decrease ($p<0.05$) of CORT–plasma levels compared to Kyo during the whole time of the experiment (Figure 4).

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Our results are in concordance with literature data about the effects of stressors activating HPA axis [31].

## 4. Discussion

Experiments about Kyo are partially connected to its` involvement in mechanisms of pain. It`s known that Kyo directly activates cortical neurons and indirectly influences μ- and δ-opioid receptors causing prolonged naloxone-reversible analgesia due to Met-Enk and β-endorphin release [24, 41]. D-Kyo has the same indirect effect on μ- and δ-opioid receptors, but causes prolonged naloxone-reversible analgesia through Met-Enk and β-endorphin release [34, 35, 53]. D-Kyo analgesia in naïve animals results to be 5.6 fold stronger than Kyo`s [41]. In our first experimental series both Kyo and D-Kyo led to decreased nociception which is consistent with literature data. The stronger analgesic effect of D-Kyo is attributed to its enzyme resistance due to replacement of L-arginine with D-arginine [41]. It`s also important that D-Kyo possesses a phenol group participating in interactions of biologically active peptides with cell membrane receptors [29, 30]. Evaluation of nociception after one hour of heat stress showed that heat stress led to higher paw pressure thresholds – results that are fully concordant with literature data about SIA. The underlying mechanism of SIA has two components – an opioid and a non-opioid one [17, 21]. The opioid component is naloxone- and naltrexone-reversible, while the non-opioid one is not sensible to μ-opioid receptor antagonists [27]. In heat-stress-induced analgesia the opioid component of SIA is the more expressed [6, 36]. It`s known that analgesic effects of heat stress are naloxone-reversible [20]. Interestingly, administration of Kyo and D-Kyo did not increase pain thresholds after heat stress. Since in the latter the opioid component is the better expressed and given the mechanisms of Kyo and D-Kyo action (through Met-Enk and β-endorphin release) it was more logic to have a potentiation of heat-stress-induced analgesia. Results showed that Kyo and D-Kyo influenced heat-stress-induced analgesia differently than our expectations. Kyo decreased the pain thresholds and shortened HP-latency for the entire time of the experiment. Pain thresholds observed were comparable with those without heat stress (as if Kyo totally "abolished" the influence of 1h HS on animals). D-Kyo decreased PP-thresholds only on the 15th min, while on the 30th min values were comparable to those after 1h HS, and on the 45th min we had even higher than after-1h HS-values. As to HP-latencies, they were shortened in respect to after-1h HS-values and also in respect to controls on the 45th min. A possible explanation of such results could be that Kyo exerts non-opioid effects, unrelated to enkephalin release [50, 53]. In fact even high Kyo concentrations in the brain steam are tightly connected with sites of opioid analgesia, yet 50% of total Kyo is in brain cortex (where opioid receptors and enkephalin concentrations are low) - suggesting a possible non-opioid pathway in Kyo`s effects. Kyo represents L-tyrosyl-L-arginine – meaning that the semi-essential amino acid L-arg is in the same time a precursor for and a metabolism product of Kyo. A dual effect of L-arg has been described: an analgesic effect through the kyotorphin-Met-enkephalin pathway and a pronociceptive one through the NO-cyclic GMP pathway [24]. In fact a pro-nociceptive effect of the dipeptidic neuropeptide has already been described in the periphery: Ueda and Inoue (2000) demonstrated that intraplantar administration of Kyo (100 fmol) elicited a nociceptive response. The effect was linked to Kyo stimulating its specific receptor, followed by Gai and phospholipase C activation [53]. Another possible explanation of our results could be that Kyo, even having analgesic effect in naïve animals, takes part of the anti-stress system decreasing stress-hormones release and increasing pain perception. Stress activates the hypothalamic-pituitary-adrenal axis causing a specific neuroendocrine pattern of interrelations in order to ensure survival and help the organism to restore homeostasis [47, 49]. Since stress is involved in the etiology and pathophysiology of different pathological condition and diseases (commonly called stress-induced diseases) [48], an anti-stress system also exists aiming at control the stress-response and avoid over-reactivity which could be deleterious to the organism [8]. Our supposition in order to explain the results obtained was that Kyo could be part of the anti-stress system of the body. The D-analog, being "unnatural" to the organism, could differently interfere with stress-answer-mechanisms and could possibly manifest its analgesic effect instead of taking part in the anti-stress system. In order to confirm such hypothesis we evaluated ACTH and corticosterone plasma levels after 1h HS followed by Kyo and D-Kyo administration.

As already described both dipeptides decreased both the evaluated hormones. ACTH plasma levels decreased to control values (on the 15th and the 30th min) and even below the control levels on the 45th min. The effects of both dipeptides were equivalent. CORT plasma levels were also decreased even without reaching the controls`. Kyo crosses the blood-brain barrier (BBB). Is transported by an H+-coupled peptide transporter PEPT2 with high-affinity and low-capacity [12, 13]. PEPT2 mRNA was reported to be strongly expressed in astrocytes of the cerebral cortex, thalamus and hippocampus [10]. Due to its vast distribution in the brain Kyo influences some neurotransmitter systems [15, 26]. It can be supposed that by modulating opioid and non-opioid neurotransmitter systems Kyo helps the organism to regain homeostasis [4, 37]. According to most of the authors D-Kyo doesn`t cross the BBB since the Kyo-transport system PEPT2 "prefers" L-amino acids [39, 46]. Nevertheless D-Kyo, similarly to Kyo binds Kyo-receptors in the peripheral tissues [7] and due to its enzyme resistance causes β-endorphin release and Met-Enk release 4 times fold compared to basal levels [34, 35, 41]. Our results demonstrated that both the dipeptides exerted the same effect on ACTH and CORT with D-Kyo having a stronger suppressive effect on the 10th min compared to Kyo. It`s known that both the dipeptides induce Met-Enk release and evidence exists that Met-Enk differently modulates other neurotransmitter systems at low and high concentrations [5]. The explanation of the results described should be sought in the involvement of other mediators – opioids, nitric oxide, serotonin, catecholamines [44]. The suppressive effects of both Kyo and D-Kyo were stronger on ACTH plasma levels than on CORT ones. We assume that the anti-stress effect of the dipeptides is better expressed in the beginning of the stress-response and effects strongly the ACTH-release phase than the CORT one. Since Kyo-synthase and Kyo-receptors have been found in rats adrenals, a direct effect of Kyo and D-Kyo on CORT could be involved [22, 54].

# 5. Conclusion

We assume that by interacting with different neurotransmitter systems after heat stress Kyo and D-Kyo take part in the anti-stress response of the body in the attempt to regain homeostasis. The authors declare no conflict of interests.

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