

Influence of the Endogenous Cannabinoid System on Antinociceptive Effects of NSAIDs Microinjected into Anterior Cingulate Cortex of Rats

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ABSTRACT. Pain is characterized as a complex experience, dependent not only on the regulation of nociceptive sensory systems but also on the activation of mechanisms that control emotional processes in limbic brain areas. Non-opioid, non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used analgesics in the treatment of not-severe pain. We have recently shown that repeated doses result in tolerance to these drugs like opioids. Here we investigated the central brain mechanisms of non-opioid induced antinociception in the non-acute pain models of rats, such as the ‘formalin test’ and a relation between administration of NSAIDs in the limbic brain area, – the anterior cingulate cortex (ACC), – and the endocannabinoid system. We measured nociceptive thermal paw withdrawal latencies and mechanical thresholds monolaterally in rats following microinjections of NSAIDs, saline or the cannabinoid receptor 1 (CB1) antagonist (AM-251) in the ACC. When pretreated with AM-251 we found a significant reduction of analgesic effects of NSAIDs (diclofenac, ketoprofen, and xefocam). The present data support the notion that endocannabinoids’ CB1 receptor contributes to antinociceptive effects of NSAIDs and probably involved in activation of the descending opioid modulatory system of pain. © 2018 Bull. Georg. Natl. Acad. Sci.

Key words: antinociception, endocannabinoids, formalin test, hyperalgesia, pain

Pain is a response of the body to the action of injuring stimuli. Notwithstanding an unpleasant experience, it appears to be an important component of the defense system of the organism and a permanent regulator of homeostatic reaction. The role of opioids in the treatment of severe pain has been long known for the humankind for thousands of years [1]. Apart from the opioid analgesics, non-opioid, non-steroidal anti-

inflammatory drugs (NSAIDs) are the most widely used analgesics in the treatment of mild pain. They elicit antinociception by action on the central nervous system (CNS) structures, besides their well-known action on peripheral tissues inhibiting cyclo-oxygenase (COX), a key enzyme in the production of prostaglandins [2]

We have recently shown that tolerance develops to analgesic effects of the commonly used NSAIDs

(metamizol, diclofenac, ketorolac and xefocam) given intraperitoneally (i.p.) in juvenile and adult rats in models of acute [3] and chronic pain (the 'formalin test') [4]. We have also revealed that repeated microinjections of these non-opioids into the dorsal hippocampus (DH) [5,6], the nucleus raphe magnus (NRM) [7], the central nucleus of amygdala (CeA) [8,9], the rostral insular cortex (RIC) [10], and the anterior cingulate cortex (ACC) [11] induce antinociception and the effects of tolerance and cross-tolerance to morphine. These findings strongly support the suggestion of endogenous opioids involvement in NSAIDs antinociception and tolerance in the descending pain-control system [1,5,9,12].

The second neuromodulatory system involved in the pathophysiology of pain that has recently raised a particular interest for the development of new therapeutic strategies is the endocannabinoids system (ECS) that plays a key role in pain control. This system is integrated by the cannabinoid receptors, their endogenous ligands, and the enzymes involved in the synthesis and degradation of these ligands [13-16]. At least 2 different cannabinoid receptors, (CB1) and (CB2), have been identified [17,18].

Experimental and clinical studies have shown the importance of the ACC in affective aspects of pain [19]. In this work we investigated the central brain mechanisms of NSAIDs antinociception in one of non-acute pain models of rats, such as the 'formalin test'. To study a relation these antinociceptive effects with endocannabinoids we treated experimental rats with CB1 receptor antagonist AM-251 in the ACC following injections with NSAIDs.

Materials and Methods

Animals. The research was carried out on adult male Wistar rats weighing 200–250 g, bred at the Beritashvili Exp. BMC. The animals were kept under standard housing conditions (22 ± 2 °C, 65% humidity, and light from 7:00 a.m. to 8:00 p.m.) and

kept on a standard dry diet with water freely available. Every effort was made to minimize both the number of animals used and their suffering. Six rats were used for each experimental and control groups. The local Bioethic Committee of the Beritashvili Center for Experimental Biomedicine approved the experimental protocols, adhering to the Guidelines of the International Association for the Study of Pain regarding investigations of experimental pain in conscious animals.

Surgical procedures. Under anesthesia with intramuscular administration of ketamine (100 mg/kg, "KharkovPharm", Ukraine), a 12-mm-long stainless steel guide cannula (Small Parts, Inc., USA) was stereotaxically implanted bilaterally into the rostral part of ACC (area I) (AP: 2.70; L: +0.5; H: 2.5) according to the coordinates in the atlas of Paxinos and Watson (1997) [20]. The guides were anchored to the cranium by dental cement. The guide cannula was plugged with a stainless steel stylet. Thereafter, the animals were handled every day for 3-4 days for 15-20 min to get familiar with the testing protocol and experimental environment. During this time, the stylet was removed and 14 mm-long stainless steel microinjection cannula was inserted into the guide cannula to reach the ACC, but no drug was injected. Five days after surgery the microinjection cannula, attached to Hamilton syringe (Hamilton, Inc., USA), was joined to the guide cannula, and the drug was introduced through it while the rat was gently restrained.

Drugs. Diclofenac (diclofenac sodium, 75 µg/0.5 µl, Hemofarm, Serbia), ketonal (ketoprofen, 25 µg/0.5 µl, Sandoz, Slovenia) or xefocam (lornoxicam, 12 µg/0.5 µl, Nycomed, Austria) were injected through the microinjection cannula as we used in previous works [5,7,10,11]. The guide cannula was then plugged with a stainless steel stylet. Isotonic saline was injected in the same volume (0.5 µl, GalichPharm, Ukraine) and manner in a separate group of rats for controls. In the second set of experiments CB1 receptor antagonist AM-251 (1 µM/0.5 µl, Sigma-Aldrich, St. Louis,

MO, USA) was injected through the microinjection cannula. Solutions were microinjected in about 10–12 seconds.

Behavioral testing. Twenty minutes post microinjection of NSAIDs or saline into the ACC, i.e. 10-min before the peak of the drugs' effect is normally reached, rats were tested for antinociception using the thermal paw withdrawal (Hargreaves) test (IITC #390, IITC Life science, Inc., Woodland Hills, CA, USA) and mechanical paw withdrawal threshold (von Frey) test (IITC Life science, Inc., USA). For Hargreaves' test Rats were first habituated over three successive daily sessions to stand on a glass surface heated to 30 ± 1 °C within a ventilated Plexiglas enclosure. Before formal testing, baseline latencies for paw withdrawals evoked by radiant thermal stimulation were measured five times per paw, with at least 5 min intervals between tests of a given paw. A light beam (Plantar Test 390, IITC) was focused onto the plantar surface of the hindpaw through a glass plate from below, and the latency from onset of the light to brisk withdrawal of the stimulated paw was measured. To prevent potential tissue damage, a cutoff time of 20 s was used if no paw movement occurred. For von Frey test baseline mechanical withdrawal thresholds were assessed using an electronic von Frey filament with 90 g range (1601C, IITC) pressed against the plantar surface of one hindpaw. This device registered the force (g) at the moment that the hindpaw was withdrawn from the filament.

In the second set of experiments, pretreatment of rats with AM-251 in the ACC was followed by thermal and mechanical tests. 10 min after they were treated with NSAIDs in the same dose as in the first set of experiments and were then retested again. Different animal groups were used for the first and second sets of experiments. The number of rats in each group was six.

Formalin-induced nociception test. Rats were placed in plastic cylinders on a room temperature glass surface and allowed to acclimate for

approximately one hour before injection. The formalin solution was prepared at 10% in saline from a formalin stock (Sigma-Aldrich, USA) and a unilateral intraplantar injection (right hindpaw) was made in a volume of 50 μ l. The formalin stock corresponded to a 37% formaldehyde solution. In rodents, intraplantar injections of formalin produce a biphasic behavioral reaction consisting of an initial phase of paw-flinching occurring about 3–5 min after the injection, followed by a quiescent period, a then second phase of flinching beginning after 20–30 min. The intensities of these behaviors are dependent on the concentration of formalin that is administered [21]. We presently collected data at minute 5 post-formalin injections representing the first phase, and at minutes 15 and 60 post-formalin injections representing the second phase.

Histology. At the end of each set of experiments, the microinjection sites were marked with 2 μ l of saturated solution of Pontamine Sky Blue (Sigma-Aldrich, USA) and the animal was euthanized with pentobarbital. After fixation by immersion in 10% formalin, the brain was sectioned and counterstained with Cresyl Violet. The microinjection sites were histologically verified and plotted according to Paxinos and Watson (1997) stereotaxic atlas coordinates [20].

Statistical analysis. All mean control and experimental groups' values are presented as mean \pm S.E.M. One-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer or Dunnett's multiple comparison tests were used for statistical evaluation of comparisons between treated and saline groups, and treated and naloxone groups, respectively. The Kolmogorov–Smirnov test was applied to verify normality. The statistical software utilized was InStat 3.05 (GraphPad Software, San Diego, CA, USA). Differences between means of saline control and treated groups, and AM-251 and treated groups of rats were acknowledged as statistically significant if $P < 0.05$.

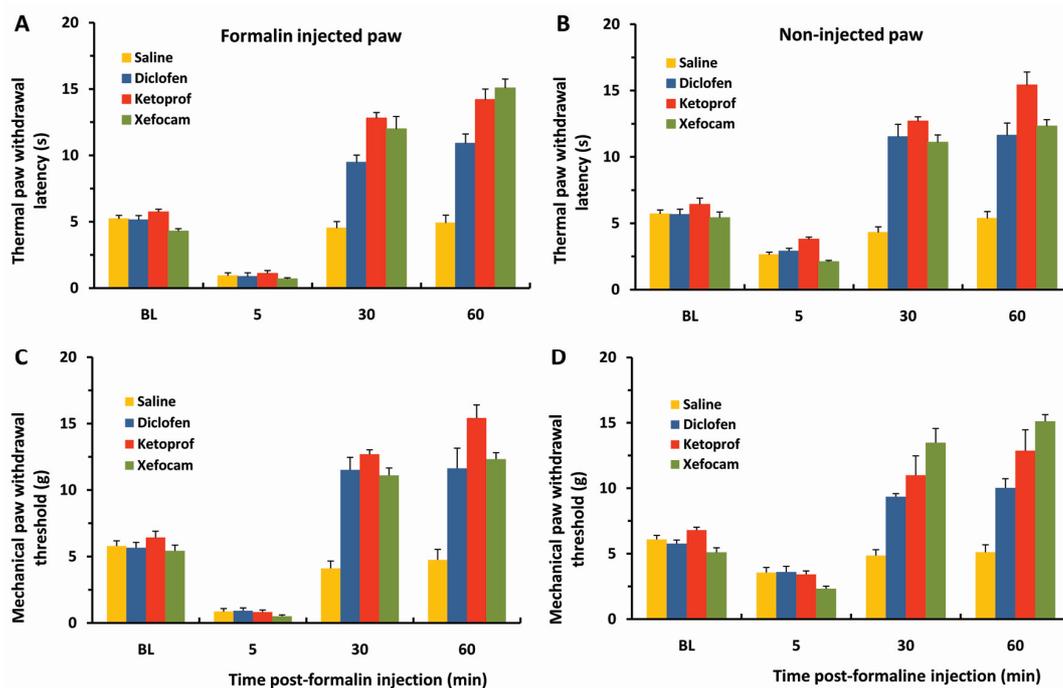


Fig. 1. Latencies of the thermal paw withdrawal reflex (s) (A, B) and thresholds of the mechanical paw withdrawal reflex (g) (C, D) after intraplantar formalin injection to one (right) paw. Note analgesics result in a significant increase in latencies and thresholds compared to the saline control for post-formalin phase II (30 min and 60 min), in formalin injected (A, C) and non-injected (B, D) paws. BL – pre-formalin baseline.

Results and Discussion

Antinociceptive effects of NSAIDs in the ACC.

The first experiment tested the acute effects of the NSAIDs on thermal and mechanical paw withdrawals during phase II post-formalin. Five min following intraplantar formalin injection (phase I), prior to the injection of NSAIDs into the ACC, all animals showed a significant reduction in thermal paw withdrawal latency and mechanical withdrawal threshold compared to pre-baseline values ($P < 0.001$) (Fig. 1A, C). These data show some spreading hyperalgesia from the formalin-injected paw to the non-injected paw ($P < 0.05$) (Fig. 1B, D).

Fifteen minutes after formalin injection, either saline, diclofenac, ketoprofen or xefocam was administered into the ACC, and thermal and mechanical paw withdrawals were assessed again

bilaterally 15 and 45 min later (i.e., at minute 30 and 60 post-formalin) during phase II. As can be seen in the saline treatment group, withdrawals recovered to near pre-formalin baseline levels. A simple comparison of pre-formalin baselines with thermal paw withdrawal latencies and threshold data at minute 30 and 60 post-formalin clearly shows antinociceptive effects of NSAIDs ($P < 0.001$).

Pretreatment with AM-251 prevents NSAIDs-induced antinociception.

In the second set of experiments, we tested if pretreatment with AM-251 would prevent NSAIDs-induced antinociception in the ACC in the post-formalin phase II. Ten minutes after unilateral intraplantar injection of formalin, rats received AM-251, followed 15 min later by microinjection of one of the NSAIDs or saline. Pretreatment with AM-251 completely prevented any thermal or mechanical antinociceptive or antihyperalgesic

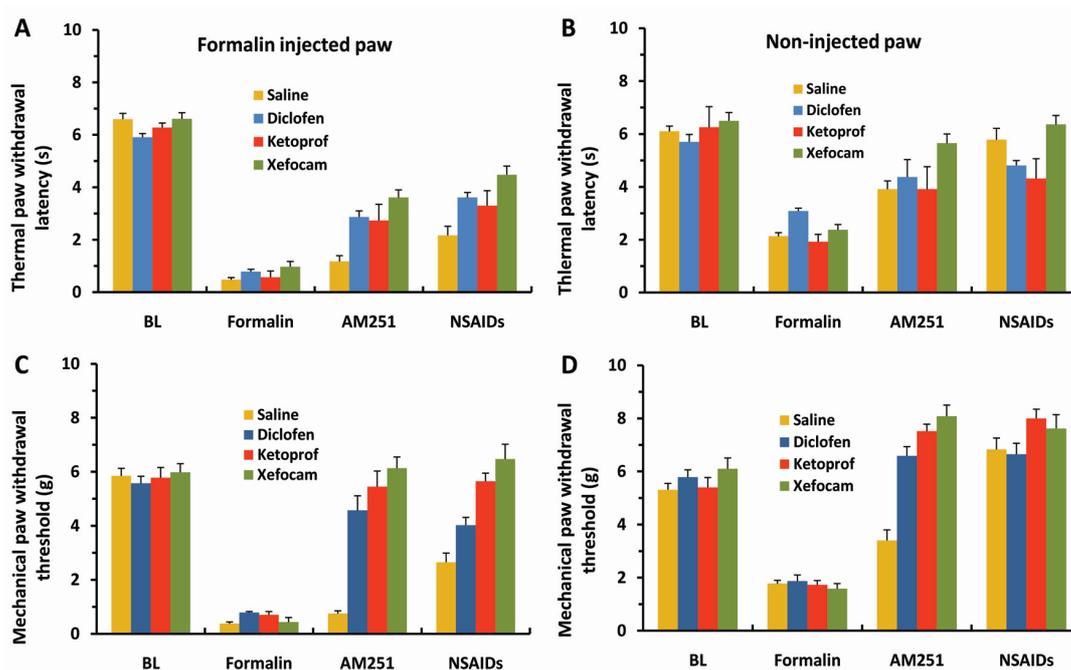


Fig. 2. Pretreatment with CB1 receptor antagonist AM-251 completely prevents analgesic effects of NSAIDs in ipsilateral (formalin injected) paw (A, C) and contralateral (non-injected) paw (B, D) in latencies of the thermal paw withdrawal reflex (s) (A, B) and thresholds of the mechanical paw withdrawal reflex (g) (C, D) for post-formalin phase II (30 min), respectively.

effect of all three NSAIDs during the phase II in the formalin-injected paw (Fig. 2 A, C). In the non-injected paw we observed almost same reduction of antinociceptive effects of all three NSAIDs in the ACC during phase II for thermal and mechanical paw withdrawal reflexes (Fig. 2 B, D).

The present study has shown that injection of commonly used NSAIDs (diclofenac, ketoprofen and xefocam) in the ACC induces antinociception in an inflammatory pain model induced by intraplantar injection of formalin into one (right) hindpaw of rats. These findings are in line with the results of our previous investigations in an acute pain model with tail-flick and hot plate tests, and in which metamizol, diclofenac, xefocam, and ketorolac were given systemically or microinjected into the periaqueductal gray matter (PAG) [2-4], into the CeA [8,12], the NRM [7,9], and the DH [5].

According to our data, CB1 receptor antagonist AM-251 completely prevented the analgesic effects of diclofenac, ketorolac and xefocam in both

ipsilateral and contralateral paws. These findings confirm previous evidence where pretreatment with AM-251 either into the lateral-ventro-lateral (LVL) PAG or into the rostral ventro-medial medulla (RVM) prevented antinociceptive effects of metamizol in Carrageenan model of hind paw inflammation of rats [22]. As authors concluded, NSAIDs might induce analgesia by acting through three mechanisms in the PAG – RVM axis. Firstly, inhibition of COXs would depress the pronociceptive effects caused by prostaglandins via the RVM. Secondly, inhibition of prostaglandin synthesis would increase the availability arachidonic acid, whose products decrease synaptic inhibition. Thirdly, by inhibiting the COXs, NSAIDs protect endocannabinoids from degradation and this also decrease synaptic inhibition [22]. As we have shown, in this pathway NSAIDs synergizes with endogenous opioids [2,5,9,12].

In the PAG – RVM axis, the action NSAIDs is reduced by the CB1 receptor antagonist AM-251.

Reduction of gamma-amino butyric acid (GABA) inhibition increases the activity PAG output neurons, which, via the RVM cause descending antinociception at the spinal cord level [22]. Taken together, these and our results suggest that descending inhibition of nociception triggered at the PAG by non-opioid analgesic, as well as by opioids, cannabinoids, GABA antagonists and other agents, depends at least

partly on endocannabinoid-induced and CB1 receptor-mediated decrease in GABAergic inhibition of spinally projecting, pain-inhibiting neurons in the RVM [12,22,23].

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(წარმოდგენილია აკადემიის წევრის თ. ზაალიშვილის მიერ)

ტკივილი წარმოადგენს რთულ ფენომენს, რომელიც დამოკიდებულია არა მარტო ნოციციცეპტური სენსორული სისტემის რეგულაციაზე, არამედ იმ მექანიზმების აქტივაციაზე, რომლებიც აკონტროლებენ ემოციურ პროცესებს თავის ტვინის ლიმბურ უბნებში. არაოპიოიდური, არასტეროიდული ანთების საწინააღმდეგო პრეპარატები (ასპ) წარმოადგენს ყველაზე ფართოდ გამოყენებულ წამლებს საშუალო სიმწვავის ტკივილის სამკურნალოდ. წარმოდგენილ შრომაში ჩვენ გამოვიკვლიეთ არაოპიოიდებით გამოწვეული ანტინოციცეპციის ცენტრალური მექანიზმები, ე.წ. 'ფორმალინის ტესტში'. ამასთან, დადგენილ იქნა კავშირი ვირთაგვების წინა ცინგულარულ ქერქში ასპ-ის შეყვანით გამოწვეულ ანალგეზიასა და ენდოკანაბინოიდურ სისტემას შორის. გაანალიზებულია თათის მოცილების თერმული რეფლექსის ფარული პერიოდი და მექანიკური სტიმულის ზღურბლი ასპ-ის, ფიზიოლოგიური ხსნარისა (კონტროლი) და კანაბინოიდური რეცეპტორის 1 (CB1) ანტაგონისტის (AM-251) შეყვანაზე წინა ცინგულარულ ქერქში. ამას გარდა, დადგენილ იქნა, რომ AM-251 წინასწარი ინიექცია იწვევდა ასპ-ით (დიკლოფენაკი, კეტოპროფენი,

ქსეფოკამი) გამოწვეული ანტინოციცეპციის სარწმუნო შემცირებას. მიღებული შედეგები ადასტურებს მოსაზრებას, რომ ენდოკანაბინოიდური რეცეპტორი (CB1) მონაწილეობს აასპ-ის ანტინოციცეპტურ ეფექტებში და, სავარაუდოდ, ჩართულია ტკივილის დაღმავალი ოპიოიდური სისტემის აქტივაციაში.

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