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METALLOTHIONEIN TREATMENT DECREASES THE HYPERALGESIA AND INFLAMMATION AFTER SURGICAL TISSUE INCISION

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The problem of postoperative pain control is still actual despite the different analgetic agents existing. In our study, we assessed the physiological, biochemical and molecular changes that develop in response to noxious peripheral stimulation after incision under metallotionein effect performed by the experimental rat model of Brennan. Post-operative hyperalgesia was persistent; the withdrawal threshold was 2.5 times less when compared to control rats 3 days after surgery and decreased to 1.5 times on day 6 after incisions. In the place of incision, the modulation of inflammation reaction was detected by an increased number of infiltrated leukocytes and high levels of proinflammatory cytokines IL-1β and TNF-α, which coincided with less MT levels in the blood. Treatment with exogenous MT by injection of 100 μg MT-II (Sigma) per animal two days before and after incision prevents the development of huge pain and inflammation on the first days after operation. We can suggest that MT may provide the zinc ions for inhibition of NMDA receptors or other signal pathway induction.

Keywords: metallotionein, post-operative hyperalgesia, pain, inflammation.

There is a lot of basic and clinical research that clear our understanding of the pain mechanism. However, postoperative pain control is still a problem despite the different analgetic agents existing. Pain from surgical incision is induced by complex stimulation including coughing, ambulation and tissue damage [10, 12]. The mechanical sensitivity of a surgical incision is the main important property that provides both peripheral and central pathways of the pain mechanisms. Damage of peripheral tissue and injury to nerves typically produce persistent pain and hyperalgesia. The peripheral component is believed to associate with peripheral inflammation due to different substances released in response to surgical trauma. That is connected with the activation of second messenger systems and triggering of the immune system. The central pathways of pain are induced through the sensitization of dorsal horn neurons by noxious stimuli. Many data provide evidences that peripheral injury produces changes in the CNS function [6]. Today we have a good characterized mechanism of neuron sensitization under pain condition [10].

However, if the peripheral inflammation caused by surgical incision plays a crucial role in postoperative pain, it is logical to speculate that synthesis induction (or additional injection) of endogenous agent that provides an antiinflammation reaction is one of the alternative ways to decrease pain without strong and long treatment by direct receptors blockader to avoid drug tolerance development.

Following this line, our interest was focused on mammalian metallothioneins (MTs), cystein-rich small metal-binding proteins that are induced not only by heavy metals (Zn, Cd) and other divalent cations (Ca, Mg, Mn), but also by specific agents such as catecholamines, glucocorticoids, interleukin-1, glucagons, tumor necrosis factor (TNF), acute phase response agents [7, 11]. Many of indicated agents take part in the peripheral and central pathways of postoperative pain. Four MT-isoforms have been characterized that have different compositions of between 61-62 amino acids and differ in localization: MT-I and MT-II - prevalent in epithelial tissue (skin, liver,

intestinal mucosa), MT-III - in the brain, MT-IV – in the squamous epithelia. The main function of MTs in eukaryotic organisms is normal zinc ion homeostasis. However, recent data demonstrate the wide range of MTs' function. It was elucidated that MT plays key role in wound repair, pathogenesis of experimental autoimmune diseases, inflammation and apoptosis pathways [3, 6, 8].

In our study, we assessed the physiological, biochemical and molecular changes that develop in response to noxious peripheral stimulation after incision under metallotionein-II effect.

Materials and methods

Wistar rats were housed by groups in standard Plexiglas cages. They were kept on a reversed 12:12 h light-dark cycle with a room temperature of 21±1o C.

Rats received treatment with i.p. injections of Zn-MT-II (Sigma, USA) or control (placebo, saline solution) treatment. The doses of MT-II treatment were previously determined to be suitable as therapy [3]. All rats were divided into 5 groups: 1- control, without any treatment (n=6); 2 and 3 – incision (n=12); 4 and 5 - injection by 100 μg MT-II per animal for 2 days before and after incision (n=12).

The experimental model of postoperative pain was developed after Brennan T. J. [2]. Operated rats were anaesthetized with ether. The plantar aspect of hindpaw was prepared in a sterile manner with iodine solution. A 1-cm longitudinal incision was made with a SM65 blade through skin and fascia of the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the heel and extending towards the toes. After haemostasis with gentle pressure, the skin was apposed with 2 mattress sutures of 3 nylons on an FS-2 needle. The wound site was covered with iodine solution and a mixture of polymixin B and neomycin ointment. After surgery, the animals were allowed to recover in the cages. The study has been approved by animal protection authorities [4].

The electrophysiological test (withdrawal thresholds) was performed with a two-pole electrode (punctuate impulses, frequency 50 Gc, duration 0.5 mc) [1]. The microelectrode was put on the non-injured foot. The test was repeated three times with a 20 min test-free period between withdrawal responses and average withdrawal thresholds (V) were presented.

The rats were sacrificed by decapitation (a half of one – on the after incision day 1; a half – on the after incision day 6). Blood was collected; serum was obtained according to standard procedure. Incised foot and the same one of the control rats were cut and fixed in Bowin solution.

The tissues were dehydrated, embedded in paraffin and cut in serial 5 μm thick sections according to standard procedures for immunohistochemistry. The sections were incubated overnight at 4o C with one of the following primary antibodies: mouse anti-human CD35 (Dako, Denmark), mouse anti-human IL-1β (Biogenesis, USA); anti-mouse TNF-α (Biosource, USA). The corresponding biotinylated second antibodies and streptavidin-peroxidase complex were used for detection (Sigma-Aldrich, USA). The positively stained cells were carried out from 0.5 mm2 matched areas of the 5 μm sections for statistical analysis. Cells were counted at the border of the incision.

The concentration of MT-I+II in the blood serum was studied by competitive ELISA using rabbit polyclonal antibody to MT-I+II and purity MT-II, zinc as a standard (Sigma, USA).

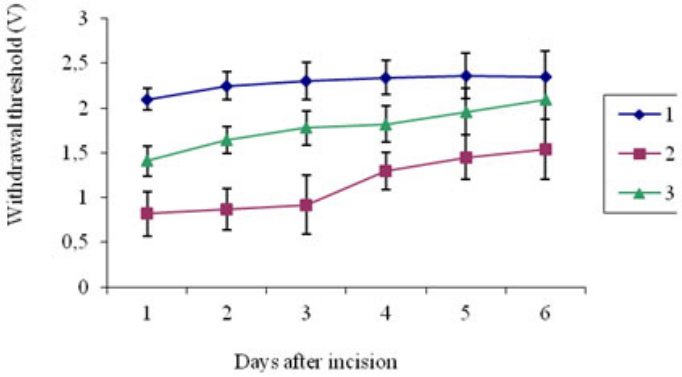
Data are expressed as the mean+SD, n=6. For statistical evaluation of the data, the Student's t-test was used. Statistical significance was taken as $P<0.05$ in all experimental groups.

Results and discussion

During experimental time, all animals were kept to maintain normal food and water intake. The weight of the control animals and rats before surgery was in average 324±25 g. There was a small significant decrease in weights during the first 3 days after incision (283-300 ± 16, $P<0.05$) for rats without additional treatment. The rats that obtained MT-treatment before and after surgery did not change in weight compared to control during the whole studied postoperative period.

Withdrawal responses to pain were estimated using electric noxious stimulus. Post-operative hyperalgesia was persistent, the withdrawal threshold was 2.5 times less when compared

to control rats through 3 days after surgery (P<0.001) and decreased to 1.5 times on day 6 after incision (P<0.05) (Fig.). Administration of 100 mg/rat of MT-II twice per day during 2 days before and after surgery prevented a strong decrease in the withdrawal threshold during the first 3 days after plantar incision and, moreover, led to a recovery studied threshold from day 5 (P>0,5).



Withdrawal threshold for rats: 1 – control rats, 2 – incision, 3 – incision with MT treatment, n=6

In order to estimate whether MT concentration in the blood serum changed under postoperative time, the ELISA method was carried out on days 1 and 6 after surgery following much alterations in algesia level through physiological tests. Obtained results indicated that the concentration of MT significantly decreased in the blood serum of rats 24 h after surgery to 0.38±0.07 μg/ml compared to 0.62±0.09 μg/ml. The level of MT in the blood serum of injured rats recovered to normal on day 6 after surgery if they obtained the injection of MT for 2 days before and after incision while rats without MT-treatment still had significant low concentrations of endogenous MT-II on day 6 after injury.

According to immunohistochemical data on day 1 after the surgical incision to the skin, we examined histopathological reactions such as inflammation. On day 1, animals showed a high number of CD-35+ leukocytes infiltrating the site of incision (Table), and the acute expression levels of proinflammatory cytokines like IL-1β and TNF-α.

Immunochemical data for the number of leukocytes, IL-1β and TNF-α positive cells at the border of the incision (N, M±m, n=6)

Groups/marker	Control	Ins-1 day	Ins-6 day	Ins+MT-1 day	Ins+MT-6 day
CD35	231,6±	463,425,7*	280,3±	293,8±	214,3±
IL-1b	121,33±	433,3±	156,2±	191±	98,5±
TNF-a	165±	339,6±	187,5±	199±	141,1±

Notes: *- P<0.001 and ** - P<0.05 compare to the control group; # - P<0.001 compare to the incision group.

In the case where animals received MT-II injections for 2 days before and after the incision, the therapeutic effect was pronounced compared to control (placebo) groups as shown on days 1 and 6 after incision (Table): the proinflammatory responses of CD35+ leukocytes and their expression of proinflammatory cytokines IL-1β and TNF-α were significantly reduced in the injured skin of MT-II treated rats relative to their controls.

In our study, we have used a rat model for acute post-operative pain due to surgical incision in the rat hindpaw. Reproducible, quantifiable mechanical hyperalgesia lasting for several days demonstrates that this model has much more similarities to the human post-operative pain state [2]. Modulation of NMDA receptors by a variety of endogenous extracellular ions and zinc is one of the main mechanisms for pain development. Previously, it was believed that zinc inhibits NMDA receptors at two independent sites: 1) low-affinity inside zinc-binding site induces the voltage-dependent inhibition of NMDA-receptors; 2) high-affinity outside zinc-binding site pro-

motes the voltage-independent inhibition. Late data suggest that tyrosine kinase Src potentiates NMDA-receptors' currents by reducing the tonic inhibition of receptors composed of NR1 and NR2A subunits by extracellular zinc]. That data link two modulatory sites of NMDA receptors.

Mechanical stress enhances the injured substances that induce the endogens defense systems. Many cellular events recognized by the system are believed to be the central events in the initiation of algesion after surgery procedure, in which products of cytokines and other metabolites play major roles too. Significant lesions were observed in the place of incision. On 1 day after incision, severe changes were characterized by the elevation in CD35+ leukocytes and their expression of proinflammatory cytokines IL-1β and TNF-α. The acute phase response is the body`s first defense against the stress, inflammation, trauma modulated by cytokine cascade including tumor necrosis factor alpha (TNF-α), interleukin-1 and increased synthesis of acute phase proteins including metallotioneins (MT). MT provides maintenance of homeostasis of zinc, which is required for the activities of more than 200 kinds of metalloenzymes and for the formation of zinc-finger motifs in transcription factors. MT can provide the zinc ions for inhibition of NMDA receptors at two independent sites noted previously. Oki G. suggested that the absence of MT from the injured peripheral nerves can play a potential pathogenic role in generating pain in the damaged peripheral nerves [10]. Application of exogenous MT before surgical incision may decrease the pain and inflammation development.

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ЛІКУВАННЯ МЕТАЛОТІОНЕЇНОМ ЗМЕНШУЄ ГІПЕРАЛГЕЗІЮ ТА ЗАПАЛЕННЯ ПІСЛЯ ОПЕРАЦІЙНОГО РОЗТИНУ ТКАНИНИ

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Проблема контролю післяопераційного болю до цього часу є актуальною, хоча й існують багато різних аналгетичних речовин. У нашій роботі за допомогою експериментальної моделі післяопераційного болю за Бренаном у щурів досліджені фізіологічні, біохімічні та молекулярні зміни, що розвиваються у відповідь на больову периферичну стимуляцію після розтину тканини та дії металотіонеїну. Стійка післяопераційна гіпералгезія супроводжувалася зниженням стійкості до електростимуляції у 2,5 разу протягом перших 3-х діб після операції та залишалася заниженою в 1,5 разу на 6 добу порівняно з контрольними щурами. У місці розтину визначено активацію запальної реакції за рахунок збільшення інфільтрованих у місце ураження лейкоцитів та збільшення вмісту прозапальних цитокінів IL-1β та TNF-α. Застосування екзогенного МТ за рахунок ін'єкцій 100 мкг МТ-II (Sigma) на тварину два дні до та після операції запобігало розвиткові сильного болю та реакції запалення у перші дні після операції. Ми можемо припустити, що МТ у змозі забезпечувати гальмування НМДА-рецепторів за рахунок донації іонів цинку або індукції інших сигнальних шляхів, що знижує розвиток больового синдрому.

Ключові слова: металотіонеїн, післяопераційна гіпералгезія, біль, запалення.

ЛЕЧЕНИЕ МЕТАЛЛОТИОНЕИНОМ УМЕНЬШАЕТ ГИПЕРАЛГЕЗИЮ И ВОСПАЛЕНИЕ ПОСЛЕ ОПЕРАЦИОННОГО РАССЕЧЕНИЯ ТКАНИ

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Проблема контроля послеоперационной боли до сих пор является актуальной, несмотря на наличие многих аналгетических веществ. В нашей работе с помощью экспериментальной модели послеоперационной боли по Бреннану у крыс исследованы физиологические, биохимические и молекулярные изменения, развивающиеся в ответ на болевую периферическую стимуляцию после рассечения ткани и действия металлотионеина (МТ). Устойчивая послеоперационная гипералгезия сопровождалась снижением устойчивости к электростимуляции в 2,5 раза в течение первых 3-х суток после операции и оставалась заниженной в 1,5 раза на 6-е сутки по сравнению с контрольными крысами. В месте разреза установлена активация воспалительной реакции за счет увеличения инфильтрированных лейкоцитов в место поражения ткани и увеличение содержания провоспалительных цитокинов IL-1β и TNF-α. Применение экзогенного МТ с помощью инъекций 100 мкг МТ-II (Sigma) на животное два дня до и после операции предотвращало развитие сильной боли и реакции воспаления в первые дни после операции. Мы предполагаем, что МТ может обеспечивать блокирование НМДА-рецепторов за счет донирования ионов цинка или индукции других сигнальных путей, что способствует снижению развития болевого синдрома.

Ключевые слова: металлотионеин, послеоперационная гипералгезия, боль, воспаление.