Intrathecal injection of GluR6 antisense oligodeoxynucleotides alleviates acute inflammatory pain of rectum in rats

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Abstract: Objective To investigate whether the kainate (KA) receptor subunit GluR6 is involved in the acute inflammatory pain. **Methods** Formalin was injected into the mucosa of rectum in Sprague-Dawley rats to induce visceral pain. The antisense oligodeoxynucleotides (ODNs) of GluR6 were injected once per day for 3 d before formalin injection, after which GluR6 protein level was examined by immunoblotting method. The change of visceral pain was also investigated. **Results** The expression of GluR6 in the spinal cord of rats increased after the formalin injection. Moreover, pre-treatment of GluR6 antisense ODNs could suppress GluR6 expression in the spinal cord of rats and decrease the scores of visceral pain at 45 min following formalin injection. **Conclusion** Kainate receptor subunit GluR6 plays an important role in the visceral pain induced by injection of formalin into the wall of rectum. GluR6 may serve as a potential target for the treatment of acute inflammatory visceral pain.

Keywords: acute inflammatory pain; rectum; GluR6; spinal cord

1 Introduction

Visceral pain is the most frequent form of pain resulting from various diseases, and it is one of the main reasons for patients seeking medical attenuation. However, the mechanisms underlying visceral pain remain poorly understood. The lack of specific treatment for visceral pain and the high prevalence of pain from internal organs have stimulated pharmacological research in new analgesic targets specifically aiming at visceral pain^[1,2].

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), where it may play important physiological roles in mediating synaptic plasticity and in cognitive functions such as learning and memory, by acting

with different groups of receptors. The ionotropic receptors have been pharmacologically divided into 3 subtypes: NMDA, AMPA and KA receptors, on the basis of their selective agonists, including N-methyl-D-aspartate, 2- amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionate, and kainate, respectively^[3-8]. So far, 5 different subunits of KA receptors have been identified, namely, GluR5, GluR6, GluR7, KA1 and KA2^[3-6,9].

Accumulating evidence has indicated that GluR6 is expressed in superficial dorsal horn and involved in nociception transmission^[10-12]. Also, recent studies have found that GluR6 is associated with peripheral inflammatory pain. For example, GluR6 immunoreactivity significantly increases in peripheral sensory axons (both unmyelinated and myelinated axons) 2 d after CFA inflammation^[11], and the expression of GluR6 is up-regulated in the spinal cord from 2 h to 3 d following CFA inflammation, which correlates well with the development of inflammation and hyperalgesia^[13]. However, little has been reported concerning the role of GluR6 in visceral pain. The present study aims to investigate whether GluR6 is associ-

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ated with visceral pain and to find new drugs targeting the visceral pain.

2 Materials and methods

2.1 Animals Adult male Sprague-Dawley rats (weighing 200-250 g) were kept under controlled environmental conditions (23-24 °C, light from 07:00 to 19:00) with free access to food and water for at least 5 d before the experiments. The surgery was performed from 09:00 to 13:00 in a noise-free room. All the experiments were approved by the local legislations on animal ethics.

2.2 Implantation of catheter For i.t. drug administration, rats should be first implanted with catheters. In brief, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), and the occipital muscles were bluntly separated to expose the cisternal membrane. Polyethylene catheter (PE-10) was inserted via an incision in the cisterna magna, and advanced 7.0-7.5 cm caudally to the level of the lumbar enlargement. Correct insertion site was confirmed by injection of 10 mL lidocaine through the catheter. The catheter was verified to be intrathecal if paralysis and dragging of bilateral hind limbs occurred within 30 s following the injection. Animals exhibiting signs of motor dysfunction were excluded from the experiment. The rats were housed separately for recovery for 5-7 d after the surgery before the following tests^[14,15].

2.3 Drug administration Intrathecal drug administration was accomplished using a microinjection syringe connected to the catheter in awake but briefly restrained rats. The injection was manually performed over a 30-s period with a single injection volume of 10 µL followed by a flush with 10 µL normal saline. Specific antisense oligodeoxynucleotides (ODNs) against GluR6 (100 mg) prepared in 10 µL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) were injected every 24 h for 3 d, through the polyethylene catheters. The same doses of missense ODNs and vehicle were used as control. The sequences of antisense and missense ODNs were as follows^[6]: antisense: 5'-CTTCATGATGTTCCGTG-3'; missense: 5'-TCTAGCTTCTGAGTTCGT-3'.

2.4 Pain behavior scores(S) The rats were anesthetized by Isoflurane inhalation, and acute inflammation of the rectum wall was induced by injection of 50 µL formalin solution (5%)^[16].

Following injection of formalin, signs of pain were scored for a total time of 45 min, which was divided into 3 sequential 15-min periods, including : (a) licking of the abdomen, (b) stretching the abdomen, (c) squashing of the lower abdomen against the floor, and (d) abdominal retractions. The pain behavior scores(S) = 1a+2b+3c+4d^[16,17].

2.5 Sample preparation The rats were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg), and the spinal cords (S2-4) were immediately removed and quickly frozen in liquid nitrogen. The spinal cords were homogenized in ice-cold lysis buffer containing 50 mmol/L 3-(N-morpholino) propanesulfonic acid (MOPS; Sigma, St. Louis, MO; pH 7.4), 100 mmol/L KCl, 320 mmol/L sucrose, 50 mmol/L NaF, 0.5 mmol/L MgCl₂, 0.2 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₃VO₄ (Sigma), 20 mmol/L sodium pyrophosphate, 20 mmol/L β-phosphoglycerol, 1 mmol/L p-nitrophenyl phosphate (PNPP), 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and each 5 µg/mL for leupeptin, aprotinin, and pepstatin A. The homogenate was centrifugated at 800 g for 10 min at 4 °C. Supernatant was collected, and protein concentration was determined by Lowry method. Samples were stored at -80 °C until use.

2.6 Immunoblotting assay Protein samples were resolved in the polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). After being blocked for 3 h in Tris-buffered saline with 0.1% Tween-20 and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 1% BSA. Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h. Protein was visualized using NBT/BCIP color substrate (Promega, Madison, WI). The protein bands were scanned and band densities were analyzed with an image analyzer (LabWorks Software, Upland, CA). β-Actin served as the internal control.

2.7 Statistical analysis Data were presented as mean±SD. Comparison was performed by one-way analysis of variance (ANOVA) with *q*-test. *P* < 0.05 was considered as statistically significant.

3 Results

3.1 The time course of GluR6 protein expression change in the spinal cord of rats after the formalin injection To observe the time course of alternation of GluR6 expression during the sigmoid wall inflammation, the spinal cords were obtained at different time points and homogenized. The expression of GluR6 protein was detected by immunoblotting. As shown in Fig. 1, the expression of GluR6 began to increase at 45 min after the injection of formalin, reaching the peak at 2 h and then gradually decreased to the level of sham group on day 7 after the injection of formalin.

Fig. 1 The time course of alteration of GluR6 protein expression in the spinal cord of rats after the injection of formalin. **A:** immunoblotting analysis of GluR6 protein level at different time points after formalin injection. β-Actin served as the internal control. **B:** quantitative representation of the immunoblotting results. Data were presented as mean±SD. **P* < 0.05 vs sham (*n* = 4 at each time point).

3.2 GluR6 antisense ODNs attenuated the formalin-induced pain behaviours in rats To find out whether intrathecal injection of GluR6 antisense ODNs could alleviate formalin-induced pain, the effects of GluR6 antisense ODNs on pain behaviours at 45 min after injection of formalin were investigated. As shown in Fig. 2, intrathecal pre-injection of GluR6 antisense ODNs could significantly reduce the scores of pain behaviours induced by injection of formalin.

3.3 GluR6 antisense ODNs reduced the protein level of GluR6 To further confirm the role of GluR6 in formalin-induced pain, the alteration of GluR6 expression was investigated using immunoblotting assay. As shown in Fig. 3, pre-

treatment with GluR6 antisense ODNs for 3 d induced a significant decrease in protein level of GluR6, compared with the injection of formalin with no pretreatment of GluR6 ODNs. Meanwhile, the same dose of vehicle or missense ODNs did

Fig. 2 Quantitative representation of the pain scores of rats at 45 min following formalin injection in the indicated groups. TE: rats with intrathecal pre-injection of vehicle. MS: rats with intrathecal pre-injection of missense ODNs against GluR6. AS: rats with intrathecal pre-injection of specific antisense ODNs against GluR6. GluR6 antisense ODNs could significantly reduce the scores of pain behaviours induced by injection of formalin. Data were presented as mean±SD. ***P* < 0.01 vs sham; #*P* < 0.01 vs AS (*n* = 8 in each group).

Fig. 3 Effect of pretreatment with GluR6 antisense (AS), missense (MS) oligodeoxynucleotides or vehicle (TE) on the expression of GluR6 at 45 min after formalin injection in the spinal cord of rats. **A:** immunoblotting analysis of GluR6 protein level. β-Actin served as the internal control. **B:** quantitative representation of the protein level of GluR6. Data were presented as mean±SD. **P* < 0.05 vs sham; #*P* < 0.05 vs AS (*n* = 8 in each group).

not affect the protein level of GluR6.

4 Discussion

A plethora of studies have demonstrated that GluR6 is involved in nociceptive transmission, and inflammatory pain could modulate the peripheral and the central expressions of GluR6 receptor^[13,18]. A recent study using antibody against GluR6 has shown that GluR6 is expressed in superficial dorsal horn in adult rats^[19]. Moreover, reverse transcription polymerase chain reaction (RT-PCR) has shown that the transcript of GluR6 is detected in the adult rat spinal cord^[13]. In spinal cord dorsal horn slices, Li *et al.*^[20] have characterized the GluR6 receptor-mediated excitatory postsynaptic current (EPSC). RT-PCR analysis indicates the increase in GluR6 mRNA expression at 2 h to 3 d following peripheral inflammation. Western blot has also shown an increase in GluR6 protein level with a time course consistent with that of the change in mRNA level^[13]. All the above studies are based on peripheral inflammation. However, whether GluR6 is involved in the acute inflammatory pain of viscera has not yet been investigated.

In the present study, the antisense ODNs of GluR6 were used to explore the role of GluR6 in the formalin-induced visceral pain model. The pain behaviors and GluR6 expression were both examined. Immunoblotting results showed that the expression of GluR6 in the spinal cord of rats increased at 45 min after the injection of formalin, and reached the peak at 2 h, recovering to normal level at 7 d, time course of which was consistent with previous research^[13]. As the pain behaviors of rats were significantly changed within 45 min after the injection of formalin, the time point of 45 min was selected to detect the effect of antisense ODNs. Results showed that pretreatment of antisense ODNs not only inhibited the expression of GluR6, but also alleviated the pain behaviors induced by formalin.

It should be noticed that the peak point of GluR6 expression was at 2 h after the formalin injection, while there was no significant behavioral sign of visceral pain in the rats. As described above, peripheral inflammation could modulate the expression of GluR6, and GluR6 participated in the peripheral inflammation-induced hyperalgesia^[13]. We therefore propose

that GluR6 may be involved in the hyperalgesia after formalin injection, which needs to be further investigated.

In summary, the GluR6 antisense ODNs can suppress the expression of GluR6 in rat spinal cord, and subsequently alleviate the formalin-induced pain. Thus, our results suggest that kainate subunit GluR6 plays a pivotal role in the visceral pain of rectum induced by formalin injection, and it may serve as a potential target in designing new therapy for visceral inflammatory pain.

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鞘内注射 GluR6 反义寡核苷酸减轻大鼠急性直肠炎性痛

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摘要: **目的** 探讨海藻氨酸受体亚单位GluR6在急性内脏炎性痛中的作用。**方法** 在SD大鼠直肠粘膜下注射福尔马林建立内脏痛模型。鞘内注射 GluR6 反义寡核苷酸, 通过行为学评分观察注射后大鼠疼痛行为学的变化, 并用免疫印迹法检测GluR6蛋白的表达变化。**结果** 直肠内注射福尔马林后大鼠脊髓GluR6表达升高。鞘内注射GluR6反义寡核苷酸后, 在福尔马林注射后 45 min 时, GluR6 蛋白表达降低, 大鼠疼痛行为学评分显著降低。**结论** GluR6 在福尔马林致大鼠直肠炎性痛中可能起重要作用, 这为临床治疗内脏痛提供了新思路。

关键词: 急性炎性痛; 直肠; GluR6; 脊髓