Effects of Combination of Ketamine with NorBNI on DREAM Protein and Ketamine-Induced Antinociceptive Effect in Formalin-Induced Inflammatory Pain

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Abstract

Downstream Regulatory Element Antagonist Modulator (DREAM) protein modulates pain by regulating *c-Fos* and prodynorphin genes transcription. The present study investigates the changes of DREAM protein expression in rat's spinal cord and ketamine-induced antinociceptive effects upon the combining administration of ketamine and nor-binaltorphimine dihydrochloride (norBNI) following formalin-induced pain. Male Sprague Dawley rats were divided into several groups: rats administered with normal saline (C), rats given only formalin injections (F), rats treated with preemptive administration of either norBNI (N+F) or ketamine (K+F) with formalin injections, and the combination of norBNI and ketamine (NK+F) administration with formalin injection. Formalin (5%) was injected subcutaneously to the rats' hind paws, and the pain behavior was recorded for one hour. After two hours, the rats were sacrificed, and their spinal cords (L4-L5) were removed for western blot analysis. *Results:* Ketamine-induced antinociceptive effect on the pain behavior responses were apparently suppressed following the combination with norBNI (NK+F). However, there was no change in spinal DREAM protein levels detected between K+F and NK+F group. *Conclusion:* The suppression effect of the ketamine-induced antinociceptive effect was attenuated when combined with norBNI, though not through the modulation of genes transcriptional mechanism regulated by the DREAM protein.

J Physiol Biomed Sci. 2017; 30(1): 19-22

Keywords: *DREAM protein; Kappa opioid receptor; NMDA receptor; pain behavior; rat spinal cord*

Introduction

There has been considerable interest in the kappa opioid receptor as a potentially new analgesic target and the endogenous kappa opioid receptor systems have been demonstrated to be involved in inhibiting hyperalgesia during inflammation.[1-2] Previous works at our lab has demonstrated that in a normal rat, preemptive ketamine administration to produce antinociceptive effects was modulated by the DREAM protein, which affected Fos and prodynorphin protein expression through their role as transcriptional repressor genes.[3] An antagonism of the N-Methyl-D-aspartate (NMDA) receptor by ketamine may result in the inhibitory effect of the kappa opioid receptor becoming more dominant and apparent. However, it is still unclear whether these effects could be due to a secondary tonic inhibition by the kappa opioid receptor and what the effect of this receptor antagonist nor-binaltorphimine dihydrochloride (norBNI) (kappa opioid receptor antagonist) on DREAM protein level.

Therefore, this study was conducted to investigate the involvement of kappa opioid receptor in ketamine-induced antinociceptive effect, using a combination of preemptive administration of nor-binaltorphimine dihydrochloride (norBNI) (kappa opioid receptor antagonist) and ketamine (NMDA receptor antagonist), on pain behavior response after formalin-induced pain. In addition, the changes of DREAM protein levels in the rat spinal cord was also investigated in order to uncover the contribution of DREAM protein on this effect.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 250-300g each were used in this study. The animals were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia and allowed to adapt for at least four days in the Physiology Department laboratory at the School of Medical Sciences. The rats were maintained on a 12-h light-dark cycle and allowed access to food and water *ad libitum*. Prior approval of the research protocol was obtained from the Animal Ethics Committee of Universiti Sains Malaysia {USM/AEG/2008/ (29) (122)}.

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Received 19 October 2016

Experimental groups

The rats were divided into 5 groups, consisting of rats that were injected with normal saline to replace formalin as a control group (C, n=6), subjected to only formalin injection (F, n=6), pretreated with norBNI (Sigma, USA) (2 mg/kg body weight i.p) with formalin injection (N+F, n=6), pretreated with ketamine (KETAVA, Atlantic labs, Thailand) (5 mg/kg body weight i.p) with formalin injection (K+F, n=6), and pretreated with a combination of both norBNI and ketamine with formalin injection (NK+F, n=6). NorBNI and ketamine were given intraperitoneally (i.p) 24 hours and 30 minutes prior to the experimental procedure. Previous studies have shown that acute norBNI administration results in a long-lasting (≥ 3 weeks) blockade of kappa opioid receptors.[4-6] Since μ opioid receptor-mediated actions of norBNI have been reported during the first 4 h after its administration; a 24 h pretreatment interval was employed.[7-8] Ketamine was given 30 minutes before formalin injection, based on our previous study.[9]

Induction of pain

The formalin injection was used as a stimulus for the induction of pain responses. The formalin test followed our previous protocol.[9]

Behavioral scoring

The rat was placed in a Perspex testing chamber measuring 26 cm x 20 cm x 20 cm. A mirror was placed below the floor of the chamber at a 45˚ angle to allow an unobstructed view of the rat's paws. Nociceptive behavior was recorded following the protocol by our previous study.[9]

Western blot analysis

DREAM protein levels in the spinal cord were analyzed using Western Blot analysis. Two hours after formalin injection, the rats were sacrificed by decapitation using a guillotine. The spinal cord tissue in the lumbar enlargement was removed directly from the rats without a fixation process being performed and separated into the ipsilateral and contralateral sides from a cut in the spinal cord at the midline. The tissue was immediately subjected to deep freeze with liquid nitrogen and kept at -80ºC until further analysis. Protein was extracted from the spinal cord tissue using the NE-PER extraction reagents (Pierce, USA). Before being used, NE-PER extraction reagents were mixed with a concentrated Halt™ Protease Inhibitor cocktail kit, EDTA-free (Pierce, USA) in a volume of 10 μl/ ml per reagent. The protein concentration of the extracted samples was measured using the bicinchoninic acid (BCA) protein assay kit. Sample protein containing 40-50 μg total protein (after optimization) were denatured and subjected to SDS-PAGE using 12% resolving gel. The protein from polyacrylamide gels was transferred to the nitrocellulose membrane (Bio-Rad, USA) using a modified technique.[10] The nitrocellulose membrane was washed with deionized water before being incubated in blocking solution (5% BSA in PBS) for 1 hour at room temperature. Following that, the nitrocellulose membrane was washed three times for 10 minutes in Tris buffer saline-Tween 20 (TBS-T20). The nitrocellulose was then incubated with rabbit polyclonal DREAM antibody (dilution 1: 500 in TBST; Santa Cruz, USA) or mouse monoclonal ß-actin antibody (dilution 1:2000 in TBST; Santa Cruz, USA) overnight at 4ºC. The nitrocellulose membrane was then incubated with HRP-conjugated goat anti-rabbit antibody (dilution 1:5000 in TBST; Santa Cruz, USA) or mouse secondary antibody (dilution 1:5000 in TBST; Santa Cruz, USA) for 1 hour at room temperature. In between incubations, the nitrocellulose membrane was washed three times in TBS-T20 for 10 minutes each. Finally, the blot was examined using Immobilon Western chemiluminescent HRP substrate (Amersham, USA) , and an image was taken using an image analyzer. The integrated density values (IDV) of the DREAM and ß-actin protein were measured using Spot Denso AlphaView™ software programmed in the image analyzer. The mean relative intensity or fold change was determined by the following formula:

Mean Relative Intensity
= (IDV DREAM protein/IDV endogenous control) target group
/ (IDV DREAM protein/IDV endogenous control) calibrator group.

Statistical analysis

The pain behavior responses were divided into 2 phases, consisting of phase 1 (mean score at 5 minutes) and phase 2 (mean scores from 15 to 60 minutes). Pain behavior responses in phase 1 and 2 were analyzed by a non-parametric Kruskal-Wallis test. When a significant value was detected, it was further analyzed by the Mann-Whitney test which was conducted for comparison between treatment groups in each phase. The DREAM protein levels were analyzed using one-way analysis of variance (ANOVA). The significant value detected was further analyzed by the *post hoc* least significance different test (LSD) for comparison between the treatment groups. All data are presented as mean ± SEM and the level of significance is set at $P < 0.05$.

Results

Pain behavior responses

The pain behavior response is found significantly increased in the NK+F group compared to the C ($P < 0.001$) group during Phase 1 (Figure 1 and Table 1). However, the effect of ketamine on the pain behavior responses is found abolished when a combination of norBNI and ketamine is administered (NK+F group). The pain behavior responses are significantly increased in NK+F group ($P < 0.01$) compared to K+F group during Phase 2 (Table 1).

Mean relative DREAM protein levels

We try using an immunohistochemistry technique to measure DREAM protein expression, but. interestingly ,no obvious cell profile was evident, and the staining revealed a punctuated pattern, making it difficult to count the number of DREAM protein neurons in each lamina (data not shown). Thus, western blot analysis was then performed to determine the levels of DREAM protein on the ipsilateral side of the spinal cord after formalin injection.

In this study, the preemptive administration of the combination of ketamine and norBNI (NK+F group) did not significantly affect the levels of the mean relative DREAM protein in the K+F group. However, the mean relative DREAM protein levels were significantly different in the K+F group compared to the N+F group ($P < 0.05$) (Figure 2A and 2B).

Discussion

There has been considerable interest in the kappa opioid receptor recently as a potentially new analgesic target. The inhibitory tone of the kappa opioid receptor system is evident when the systemic

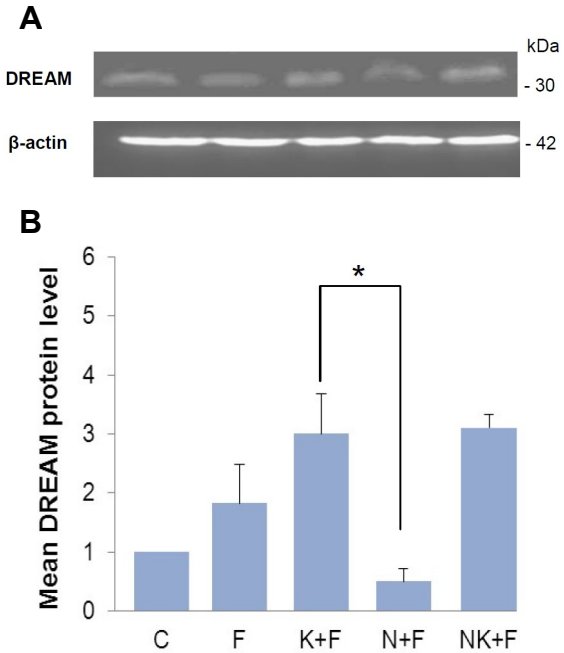

Figure 2 A representative example of western blot results for nuclear extracts on the ipsilateral side between all groups with quantification analysis of the integrated density value (A). Columns represent the mean relative DREAM protein level ± SEM for six separate experiments (B). The mean relative DREAM protein level (fold change) represents comparative levels of DREAM protein in the experimental groups (formalin injected group, F, ketamine and formalin injected group, K+F, norBNI and formalin injected group, N+F, combined norBNI with ketamine and formalin injected group, NK+F) over the calibrator group (control group, C) after normalization by its loading control (housekeeping protein, β-actin protein) at 2 hours after formalin injection. n = 6 for each group. *$P < 0.05$ compared between K+F and N+F groups.

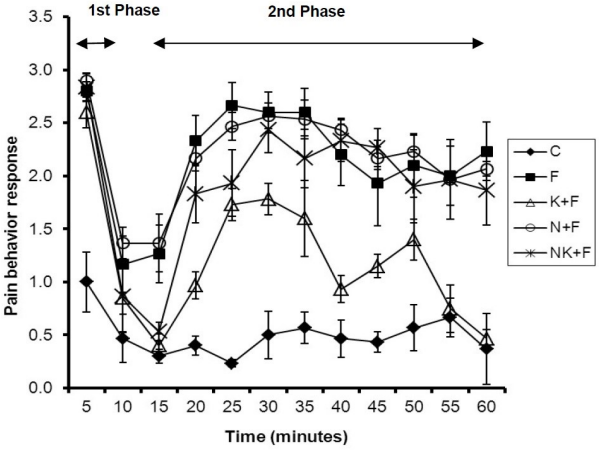

Figure 1 Pain behavior response for all groups in 1 hour periods. Values are mean ± SEM; n = 6 for all groups.

Table 1 Pain behavior response during phase 1 and 2.

Group	Pain behavior response	
	1st Phase	2nd Phase
Control	1.0 ± 0.28	0.5 ± 0.03
F	2.8 ± 0.09***	2.2 ± 0.22**
K+F	2.6 ± 0.15***	1.1 ± 0.06
N+F	2.9 ± 0.07***	2.2 ± 0.04**
NK+F	2.8 ± 0.13***	1.9 ± 0.20**

Values are mean ± SEM; n = 6 for all groups. ***$P < 0.001$ compared to Control; **$P < 0.01$ compared to K+F groups.

administration of the kappa opioid receptor antagonist, norBNI was shown to increase the flinching behavior in rats during the tonic phase of formalin test compared to formalin injected group.[1] However, in this study, we found that the ketamine-induced antinociceptive effect was suppressed when a combination of norBNI and ketamine was administered (NK+F group). The preemptive administration of the combination of norBNI and ketamine has been found to further increase pain behavior response if compared to norBNI administration alone in the second phase of formalin test, associated with an increased Fos protein expression in the spinal cord that appears to decrease in the group that received preemptive administration of ketamine alone (K+F group).[3] The finding in the NK+F group indicates that there is no certain correlation or link existing between the mechanisms of action of both the kappa opioid and NMDA receptors in the modulation of formalin-induced inflammatory pain response. It suggests that no involvement of kappa-opioid receptor activation in the ketamine (NMDA receptor antagonist) induces the antinociceptive effect. The mechanism for this effect is still uncertain, but it has been proposed that this effect could be related to the changes of Fos and prodynorphin protein expression in the spinal cord.

Previous work at our lab has demonstrated that ketamine-induced antinociceptive effects are modulated by DREAM protein, which can affect Fos and prodynorphin protein expression in the spinal cord through their role as a transcriptional repressor during formalin test.[3] In this study, we found that the effect

of preemptive administration of norBNI (N+F group) alone on DREAM protein levels was no longer present in the nuclear extract in the spinal cord after the combination of norBNI and ketamine (NK+F group) was administered. The DREAM protein levels in NK+F group tended to increase if compared to the N+F group. However, DREAM protein levels in the NK+F group were found to not significantly change if compared to the K+F group. It seemed that an antagonism of both the kappa opioid and NMDA receptors (NK+F) reversed the effects of preemptive administration of norBNI (N+F) but not ketamine (K +F) on DREAM protein levels in the spinal cord. From the results, it is suggested that the suppression of the ketamine-induced antinociceptive effect after administering a combination of norBNI and ketamine possibly did not involve the DREAM protein changes that modulated the transcriptional processes of the *c-Fos* and prodynorphin genes in rat spinal cord.

In conclusion, the results in this study suggest that the kappa opioid receptor was not involved in suppressing the ketamine-induced antinociceptive effect secondary to the combined administration of norBNI and ketamine, and not regulated by DREAM protein that modulated the *c-Fos* and prodynorphin genes transcriptional mechanism.

Acknowledgments

This study was supported by the Research University Grant Scheme [1001/PPSK/812022] and Postgraduate Research Grant Scheme [1001/PPSK/8142001].

Conflict of Interest

None to declare.

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