Full-length article

Intrathecal administration of roscovitine inhibits Cdk5 activity and attenuates formalin-induced nociceptive response in rats[1]

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Key words

roscovitine; cyclin-dependent kinase-5; DARPP-32

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Abstract

Aim: To investigate effects of the cyclin-dependent kinase5 (Cdk5) inhibitor roscovitine on formalin-induced nociceptive responses in rats. **Methods:** The flinch response as a methood of pain threshold measurement and intrathecal injection techniques were used. Cdk5 and phosphorylation of its downstream target, DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa), were investigated by Western blot analysis. **Results:** Rats demonstrated a typical flinch response after formalin injection. Intrathecal roscovitine injections significantly suppressed the flinch response in a dose-dependent manner. Western blot analysis showed that phosphorylated DARPP-32 at Thr75 increased in concentration after formalin hyperalgesia, with this effect reduced by roscovitine administration. This antinociception was partially attenuated by administration of naloxone before the formalin test. **Conclusion:** DARPP-32 phosphorylation is involved in acute inflammatory pain response. Intrathecal roscovitine administration attenuates formalin-induced nociceptive responses and there is potential for further application.

Introduction

Tissue injury is associated with sensitization of nociceptors and subsequent changes in the excitability of central neurons, known as central sensitization. Nociceptor sensitization and central sensitization are believed to underlie the development of primary and secondary hyperalgesia[1]. Glutamate, acting at the spinal *N*-methyl-*D*-asparate (NMDA) receptor, has been implicated in the development of secondary hyperalgesia[2,3]. Downstream of NMDA receptor activation, spinal nitric oxide (NO), protein kinase C, and other mediators have been implicated in maintaining such hyperalgesia[4]. Among these mediators, cyclin-dependent kinase-5 (Cdk5) has been found to be involved in modulation of the NMDA and metabotropic glutamate receptors[5].

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa) is a cytosolic protein that is selectively enriched in medium spiny neurons in the neostriatum[6]. When DARPP-32 is phosphorylated by cAMP-dependent protein kinase (PKA) on Thr34, it is converted into a potent inhibitor of protein phosphatase-1 (PP-1)[7]. This leads to an increase in the phosphorylation of downstream PP-1 substrates, including various neurotransmitter receptors and voltage-gated ion channels[6]. If DARPP-32 is phosphorylated at Thr75 by Cdk5, it inhibits PKA activity and thereby reduces the efficacy of dopamine signaling[8]. Thus, DARPP-32 is a bi-functional signal transduction molecule that controls the activity of PP-1 and PKA by phosphorylation at different sites.

Roscovitine is a potent selective inhibitor of Cdk5, and competes for the ATP-binding site of the kinase (ED_{50}=0.16 μmol/L)[9]. Injection of roscovitine inhibits Cdk5 activity in the hippocampus and reduce the NMDA-evoked currents[10]. Recently, we demonstrated roscovitine mediated antinociception and attenuated morphine tolerance in rats[11]. Therefore, in the present study, the effect of roscovitine on the nocice-

ptive flinch response, evoked by subcutaneous formalin injection, and its possible molecular mechanism, was investigated.

Materials and methods

Drug Roscovitine was obtained from LC Laboratories (Woburn, MA, US) and Me_2SO was purchased from Sigma-Aldrich (St Louis, MO, USA).

Animal care and intrathecal catherterization Male Sprague-Dawley rats (250–300 g) were provided by the "National" Science Council, Taiwan, China. The rats were housed in a room with a 12:12 h dark-light cycle, and a temperature of 22±0.5 ºC, with food and water *ad libitum*. The ethical guidelines specified by the Chang-Gung Memorial Hospital Animal Ethics Committee were followed throughout the study. Chronic intrathecal catheters were implanted under isoflurane anesthesia. Through an incision in the atlanto-occipital membrane, a polyethylene (PE-5) catheter, filled with 0.9% saline, was advanced 8.5 cm caudally to position its tips at the level of the lumbar enlargement. The rostral tip of the catheter was passed subcutaneously, externalized on top of the skull, and sealed with a stainless-steel plug. Rats showing neurological deficits after implantation were excluded from the study. Rats were used for experimentation three days after implantation.

Behavioral testing and animal grouping For formalin injection, 50 μL of a 5 % formalin solution was injected subcutaneously into the dorsal surface of the right hind paw using a 27-gauge needle. Animals were then placed in a clear Plexiglas cylinder (30 cm×30 cm) for observation. A mirror was placed below the floor (Plexiglas) at a 45º angle, to enable unencumbered observation during the test. Within 1 min of the injection, the rats displayed the typical behavior of this model, holding the paw just off the floor. During this period, spontaneous flinching of the injected paw was also observed. Flinching was readily discriminated, and was characterized as a rapid and brief withdrawing or flexing of the injected paw. Pain-related behavior was quantified by counting the number of flinches over 1 min intervals during the first 5 min, and then at 5 min intervals 10–60 min after formalin injection. Two phases of spontaneous flinching responses were observed. An initial acute pain response (phase I, during the first 1–5 min after formalin injection) was followed by a relatively short quiescent period and then by a prolonged tonic phase (phase II, beginning approximately 10–60 min after formalin injection). Criteria for exclusion from the study included incomplete formalin injection, or excessive bleeding from the injection site. Time-response data was presented as the mean±SD per minute for the period of 1–9 min, and then at 5 min intervals up to 60 min. For the dose-response analysis, data from phase I and phase II observations were considered separately. In each case, the observation interval was calculated for each rat. The cumulative flinching response was calculated for each animal, and the dose-response curve represents the mean±SD. To determine the dose dependency and time course of the antinociceptive action of an intrathecal injection of roscovitine, animals were randomly assigned to five groups receiving different doses of roscovitine: 0, 10, 50, 100, 200 μg (n=6 in each group), injected intrathecally 30 min before formalin administration. For exploring possible μ-opioid involvement in the effect of intrathecal roscovitine, naloxone (1 mg/kg) was given intraperitoneally 1 h before the formalin test in the 200 μg roscovitine group (n=6). Roscovitine was dissolved in dimethyl sulfoxide (Me_2SO) and delivered with a microsyringe in a total volume of 10 μL, followed immediately by 5 μL of Me_2SO to flush out the catheter.

Western blot analysis One hour after formalin injection, the rats were killed under deep isoflurane anesthesia, then decollated and spinal cord was removed. The lumbar spinal cords were homogenized in a lysis buffer (20 mmol/L Tris pH 7.6, 150 mmol/L NaCl, 1 mmol/L EGTA, 5 mmol/L NaF and 1 mmol/L dTT, supplemented with protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and complete phosphatase inhibitors. For analysis of Cdk5 and DARPP-32 protein expression after the formalin test, 25 μg protein extracts were electrophoresed on a 12% acrylamide SDS polyacrylamide gel electrophoresis and immunoblotted onto polyvinylidene fluoride membranes. The membranes were blocked for 1 h at room temperature and incubated overnight with Cdk5 (C-8) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), DARPP-32 (1:1000) (Cell Signaling Technology, Beverly, MA, USA), phospho-DARPP-32 (Thr-75) antibody (1:500) (Cell Signaling Technology), and α-tubulin antibody (1:1000) (Santa Cruz Biotechnology) was used as an internal control. Antibody binding was detected using a goat anti-rabbit horseradish peroxidase-linked IgG. The bands were visualized by an ECL detection system (Amersham-Pharmacia Biotech, Little Chalfont, England). Band intensities were quantified by using an image analyzer (Densitograph AE-6900M, Atto, Tokyo, Japan).

Statistical analysis All the data in this study are presented as means±SD and analyzed by one-way ANOVA followed by Dunnett's test for *post-hoc* analysis. P<0.05 was considered significant.

Results

Roscovitine attenuated the formalin-induced flinch response Formalin (5%, 50 μL) injected into the dorsal surface of a rat hind paw produced characteristic behaviors. The intrathecal administration of 0, 10, 50, 100, and 200 μg roscovitine produced a dose-dependent decrease in the flinch response to formalin (Figure 1). The cumulative phase I flinch counts were 79±6, 81±4, 60±7, 60±6, and 23±2, respectively. There was a statistically significant difference between the 200 μg group and the 0 μg group (P<0.01). The cumulative phase II flinch counts were 117±6, 112±7, 65±25, 46±11, and 16±2. The 50, 100, and 200 μg groups all showed significant inhibition of the phase II flinch response compared to the 0 μg group (P<0.01).

Naloxone reversed the roscovitine mediated antinociception Another six rats were given naloxone (1 mg/kg) 1 h before the formalin test in the 200 μg roscovitine group. The cumulative phase I flinch count was 48±5 and the phase II flinch counting was 92±10. The difference in phases I and II were statistically significant compared to the 200 μg group (P<0.01) (Figure 1).

Roscovitine attenuated the phosphorylated DARPP-32 upregulation after formalin hyperalgesia Western blot analysis showed that the Cdk5 expression did not change significantly in any groups, and neither did the total DARPP-32, the downstream target of Cdk5 (Figure 2). However, the phosphorylated-DARPP-32 at Thr-75 was upregulated significantly after formalin hyperalgesia compared to the sham group, which did not receive formalin injection on the paw. Intrathecal roscovitine attenuated the increase in the proportion of phosphorylated DARPP-32 proportion significantly as compared to the 0 μg group [25±6, 10±5, 15±6, 20±7, and 10±4 (arbitrary percentage calculated by phosphorylated-DARPP-32 Thr-75/DARPP-32) in 10 μg , 50 μg, 100 μg, 200 μg, and sham groups respectively after three separate experiments, P<0.01] (Figure 3).

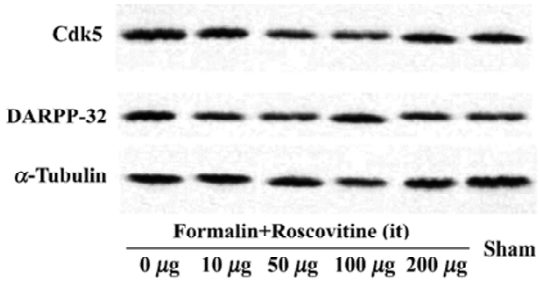

Figure 2. Western blot analysis of the effect of intrathecal roscovitine administration on Cdk5, total DARPP-32, and α-tubulin after the formalin test. α-Tubulin was used as an internal control. There was no significant change in Cdk5 and total DARPP-32 between groups.

Discussion

The formalin test is a model that is believed to underlie abnormal pain perception in humans following injury[12,13]. Following subcutaneous injection of formalin into the hind paw of a rat, the animal displays spontaneous pain behavior,

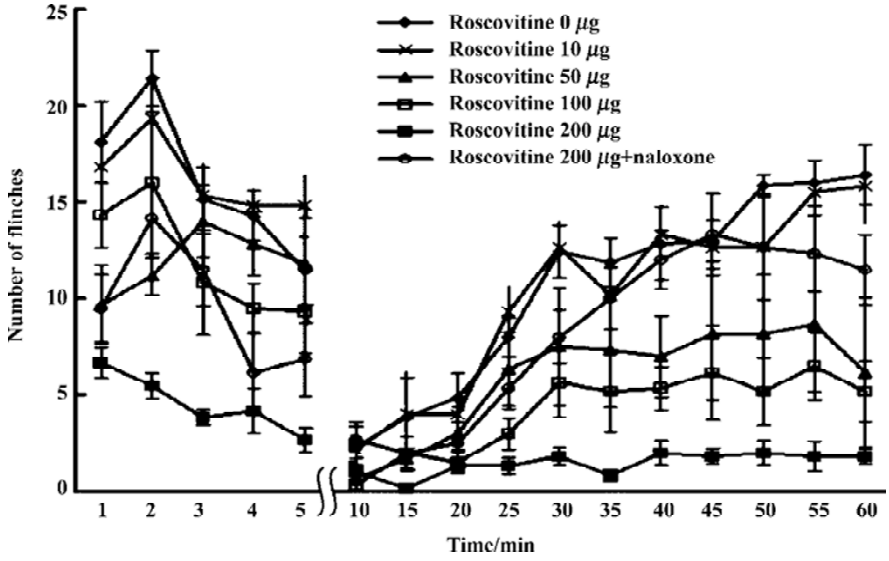

Figure 1. Number of flinches per minute in the formalin test over time, assessed after intrathecal administration of the Cdk5 inhibitor roscovitine. Mean±SEM. n=6.

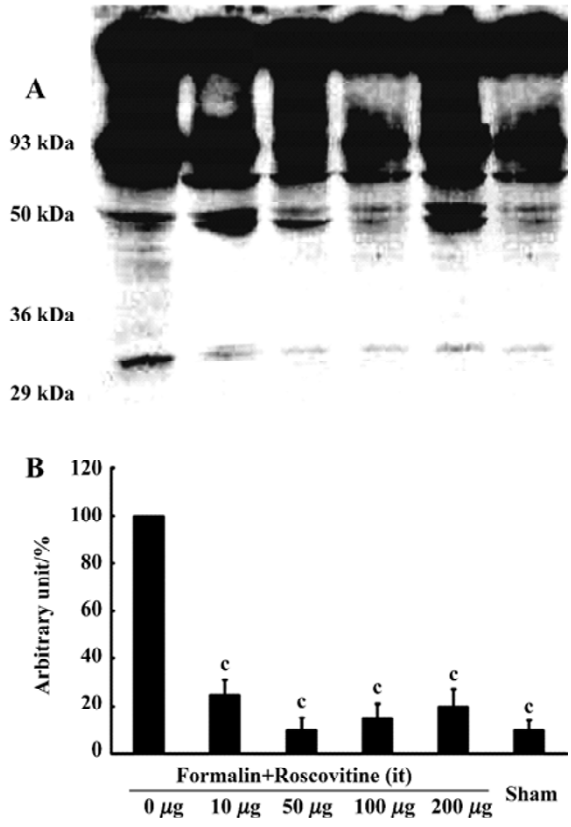

Figure 3. A) Immunobloting of phosphorylated DARPP-32 at Thr-75. B) Quantitative analysis of the proportion of phosphorylated DARPP-32 at Thr-75 after formalin hyperalgesia. Intrathecal roscovitine attenuated the increase of phosphorylated form with statistical significances as compared to the 0 μg group ($P<0.01$ *vs* 0 μg group. Three separate experiments). Arbitrary unit (100 %) was defined as percentage of phosphorylated DARPP-32 in the formalin+roscovitine 0 μg group.

that is, increased hind paw flinching and licking[14]. Two distinct phases were observed, an early acute phase (phase I) followed by a later tonic phase (phase II). The response to formalin during the acute phase is attributed to the pain intensity itself. The response during tonic phase is believed to be mediated through increased spontaneous activity of the spinal cord dorsal horn neurons[15].

Cdk5 is a member of the Cdk family of serine/threonine kinases. Recently, both NMDA and metabotropic glutamate receptors have been shown to be modulated by Cdk5[5]. Previous experiments have shown that inhibition of Cdk5 activity in hippocampal CA1 neurons by roscovitine injection results in a reduction of long-term potentiation and NMDA-evoked currents[10]. Cdk5 may be one of the most important kinases in the regulation of neurotransmitter release. The induction of neurotransmitter release by Cdk5 inhibitors is caused by the regulation of P/Q-type voltage-dependent calcium channel activity[16]. Therefore, Cdk5 might play an important role in nociception by controlling neurotransmitter release.

In this study, intrathecal roscovitine reduced the flinch response after formalin hyperalgesia in a dose-dependen manner. Western blot analysis revealed that the levels of phosphorylated DARPP-32 at Thr-75 were increased after formalin hyperalgesia, and this upregulation was blocked by intrathecal roscovitine administration. Hence, Cdk5 may affect this inflammatory pain model through the DARPP-32 pathway.

Interestingly, naloxone markedly decreased intrathecal roscovitine-induced antinociception, suggesting the involvement of μ-opioid receptors. It is noteworthy that naloxone did not completely block roscovitine-induced antinociception. The exact underlying mechanism is not clear. However, it might suggest that the μ-opioid receptor and Cdk5 are integrated in the DARPP-32 pathway as reported by Greengard[6].

This is the first study exploring the antinociceptive effect of intrathecal roscovitine on formalin-induced pain. Our findings suggest an induction of phosphorylated-DARPP-32 at Thr-75 after formalin hyperalgesia. Intrathecal roscovitine administration attenuated these flinch responses, at least in part, through inhibition of Cdk5 activity. Taken together, these data suggest that spinal Cdk5 activity might play an important role in nociception, and that its inhibitor, roscovitine, could be applied in the management of acute inflammatory pain.

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