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Attenuation of neuropathic pain and neuroinflammatory responses by a pyranocoumarin derivative, anomalin in animal and cellular models

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ABSTRACT

The present study investigated the neuropathic pain, anti-neuroinflammatory and neuroprotective properties of a pyranocoumarin derivative (anomalin) in in vivo and in vitro models. An in vivo streptozotocin (STZ)-induced diabetic neuropathic pain model demonstrated that anomalin significantly suppressed neuropathic pain in mice. To identify the molecular mechanism of the anti-neuropathic pain activity of anomalin, sodium-nitroprusside (SNP)-induced neuroinflammation in neuro-2a (N2a) cells was further investigated in signaling pathways. The effects of anomalin against SNP-induced toxicity, nitrite production and related mRNA gene expression (iNOS and COX-2) were considerably reduced by anomalin in the SNP-induced N2a cells. In the molecular signaling pathway, anomalin effectively blocked the SNP-induced activation of the IKKα/β, IκBα, ERK1/2 and p38 MAPK pathways. Furthermore, anomalin remarkably reduced the increase in the SNP-induced nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway. Additionally, the pro-inflammatory cytokines level was remarkably inhibited by anomalin in high glucose-induced DRG primary neurons and SNP-induced N2a cells. These findings indicate that anomalin has anti-neuropathic pain, anti-neuroinflammatory and neuroprotective effects against STZ-induced diabetic type I neuropathic pain and SNP-induced in neuronal cell models via the inactivation of the NF-κB, Nrf2 and MAPK signaling pathways.

1. Introduction

Neuropathic pain results from the damage of peripheral nerves; it is often persistent and is poorly treated by the existing therapies because distinct mechanisms are triggered by neuronal injury (DeLeo et al., 2004). Peripheral nerve injury because of trauma, disease, and certain toxins may produce abnormal (neuropathic) pain syndromes that are chronic and refractory to analgesic agents (DeLeo et al., 2004). Neuropathic pain can arise from nerve damage, including diabetic neuropathy, HIV neuropathy, post-herpetic neuralgia, drug-induced neuropathy and traumatic nerve injury. In damaged peripheral nerves, macrophages are recruited by chemotactic molecules, and other evidence indicates that macrophages are important in neuropathic pain models (Finnerup et al., 2005; Le Bars et al., 2001; Marchand et al., 2005).

Numerous reports have demonstrated that peripheral nerve damage injury produces long-lasting, heterogeneous pain conditions referred to as neuropathic pain. Experimental models of peripheral nerve injury in animals have been developed that produce behaviors suggestive of mechanical allodynia and thermal hyperalgesia, which correspond, to some degree, to a clinically relevant neuropathic pain model (Milligan and Watkins, 2009). The field of neuropathic pain has been greatly advanced by the introduction of animal models of post-traumatic painful peripheral neuropathy with comprehensive understandings of the mechanisms that produce neuropathic pain and the ability to identify new anti-neuropathic agents. Though these models are most clearly relevant to peripheral neuropathies (e.g., causalgia), they are also likely related to painful peripheral neuropathies evoked by disease (e.g., diabetes) and toxins (e.g., chemotherapeutics).

The development of therapeutics for the treatment of chronic illness has been particularly challenging. Clinical trials have identified effective drugs to treat chronic inflammation and pain (Finnerup et al., 2005), such as opioids, anti-epileptic agents, and anti-depressants. Interestingly, none of these agents was developed to treat chronic inflammation and pain (Brill, 2013a, 2013b; Vinik and Casellini, 2013). To date, all of the drugs available for chronic pain treatment have failed with respect to the

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effectiveness and safety. The development and utilization of more effective anti-inflammatory and analgesic agents of natural origin remain desired and more challenging.

Previous findings have also demonstrated that anomalin inhibited the nociception induced by CFA- and carrageenan-induced animal models. Because of nociceptive effect of anomalin, we aimed to explore the possibility of investigating the neuroprotective property, anti-neuroinflammatory mechanism and effect of anomalin in a neuropathic pain model. Therefore, a STZ-induced diabetes mellitus animal model and sodium nitroprusside (SNP)-induced neuroblastoma cells (N2a) in vitro were utilized. Additionally, attempts have been made to investigate the potential mechanisms of anti-neuroinflammation and anti-oxidants through which anomalin exerted its effects.

2. Materials and methods

2.1. Animals

Male ICR mice (Samtako, Osan, Korea), 3–4 weeks of age, weighing 30–35 g, were used in the present study. All animal studies were performed in a pathogen-free barrier zone of the Seoul National University Animal Laboratory, according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals (Seoul National University, Korea). The animals were housed at 23 ± 0.5 °C with 10% humidity in a 12 h light–dark cycle. Animal care and handling procedures were followed according to the guidelines of the International Association for Study of Pain (IASP) on the use of animals in pain research (Zimmermann, 1983). Behavioral tests were done without knowing to which experimental group each mouse belonged. Each group contained five mice. All animals were used only once. Every effort was made to minimize the number of animals used and any discomfort. The animals were divided into three groups (control; saline with 10% DMSO, 50 mg/kg celecoxib, 50 mg/kg anomalin).

2.2. STZ-induced animal model and measurement of mechanical allodynia

Diabetic neuropathic pain was modeled using the antibiotic drug streptozotocin, which targets and kills the pancreatic β islet cells and induces type I diabetes in animals. A STZ-induced neuropathic model was performed according to previously described methodology (Wagner et al., 2013). Briefly, diabetes mellitus type I was induced by intraperitoneal injections of streptozotocin (STZ, 150 mg/kg freshly dissolved in sodium citrate buffer at pH 4.5). All animals were fasted 6 h prior to STZ injection. Ten percent sucrose in water was supplied overnight to avoid sudden hypoglycemia after STZ injection. Diabetes was allowed to develop and stabilize in these STZ-treated mice over a period of 5–7 days. After 6–7 days, the blood glucose level was measured from the tail vein according to previously reported methodology (Cho et al., 2012; Ojewole, 2008). Mechanical allodynia was measured according to Cho et al. (2012). For the Von Frey test, mice were placed individually in a transparent plastic box with a mesh floor to allow access to the ventral surface of the hind paw. Prior to each testing session, the animals were habituated to the testing environment for at least 30 min. Mechanical hypersensitivity was assessed using calibrated Von Frey filaments (Stoelting, USA) by those blinded to the group assignments. Each filament was applied five times to the plantar surface of the right hind paw in an ascending order of force. The withdrawal reflex on at least three of the five applications was defined as a positive response. To evaluate the drug effects, anomalin (50 mg/kg) or celecoxib (50 mg/kg) was administered by intraperitoneal (i.p.) routes daily for 5 consecutive days, and the control group only received the vehicle. Mechanical allodynia was measured every day after 4 h of treatment.

2.3. Acetone test

Acetone test was performed for the evaluation of cold pain (allodynia) on mice hind paws. Mice were placed on a metal mesh floor with small Plexiglas cubicle containers and allowed at least 30 min for habituation before testing. Total duration of acetone evoked behaviors (flinching, licking or biting of their hind paws) was counted during 1 min after one drop of acetone (~25 μl) application to the plantar surface of the hindpaw.

2.4. Primary DRG neuron cultures

Primary cell cultures of DRG neurons were conducted as previously described (Cho et al., 2012). Thoracic and lumbar DRGs were dissected from wild-type mice, and collected in cold culture medium (4 °C) containing a mixture of DMEM and F-12 solution, 10% (vol/vol) fetal bovine serum (Gibco BRL), 1 mM sodium pyruvate, 50–100 ng ml^{-1} nerve growth factor and 100 units per ml of penicillin/streptomycin. Ganglia were washed with culture medium and incubated for 30 min in a warm (37 °C) DMEM/F-12 mixture containing 1 mg ml^{-1} collagenase (Type II, Worthington Biomedical). DRGs were then washed three times with Mg^{2+} and Ca^{2+} free Hank's solution, and incubated with gentle shaking in Hank's solution containing 2.5 mg ml^{-1} of trypsin (Roche Diagnostics) for 30 min at 37 °C. The trypsin-containing solution was then centrifuged at 100 g for 10 min. The pellets so obtained were washed gently two to three times with culture medium, suspended in culture medium, gently triturated with a fire-polished Pasteur pipette and plated onto round glass coverslips (Fisher), which had been previously treated with poly-L-lysine (0.5 mg ml^{-1}), in small Petri dishes (35 × 12 mm). Cells were then placed in a 37 °C incubator in a 95% air/5% CO_2 atmosphere. Cells were used 2–4 days after plating.

2.5. Cell culturing, cell viability and NO assay

The mouse neuroblastoma cell line, neuro-2a (N2a), PC-12 and RAW 264.7 cells were obtained from ATCC (MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The PC-12 cells were cultured in F12 media (Ham's Nutrient Mixture F12; Sigma-Aldrich, St. Louis, USA). Briefly, the N2a, PC-12 and RAW264.7 cells were plated at a density of 1×10^5 per well in a 24-well plate and incubated at 37 °C for 24 h. After an overnight culture, the medium was replaced with fresh medium without FBS. To induce cell injury, the cells were incubated with 500 μM SNP (Sigma-Aldrich, St. Louis, USA) for 20 h. To identify the effects of anomalin, the cells were pre-incubated with anomalin for 24 h, and SNP was subsequently added to the medium for an additional 24 h. After incubation for 24 h, 100 μl aliquots of the cell-free culture medium were taken for NO measurement according to the Griess reaction method, and cell viability was measured as previously described (Khan et al., 2011). *N-p*-tosyl-l-phenylalanyl chloromethyl ketone (TPCK 30 μM) was used as a positive control.

2.6. Quantitative real-time (qRT)-PCR

RT-PCR was performed with total RNA extracted using easy-Blue™, according to the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO). The purity and concentrations of RNA were determined using the ND-1000 spectrophotometer (Nanodrop