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ORIGINAL ARTICLE

Promotion of thermal analgesia and neuropeptidergic skin reinnervation by 4-methylcatechol in resiniferatoxin-induced neuropathy

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Abstract To investigate whether 4-methylcatechol (4MC) could decrease the duration of the thermosensation disorder and promote the innervation of peptidergic intraepidermal nerve fibers (IENFs), we developed a resiniferatoxin (RTX)-induced neuropathic mouse model with thermal analgesia and skin denervation that was followed by daily 4MC treatment. On day 7 after RTX administration (RTXd7), the substance P (SP)(+) IENFs were completely depleted compared with the vehicle group ($p < 0.0001$), whereas the calcitonin gene-related peptide (CGRP)(+) IENFs were dramatically, but not completely, depleted ($p < 0.0001$). While SP(+) IENFs remained depleted ($p = 0.0043$), CGRP(+) IENFs were recovered by RTXd84 ($p = 0.78$). 4MC had no effect on the reinnervation of SP(+) IENFs, but markedly promoted the reinnervation of CGRP(+) IENFs on RTXd35 ($p = 0.035$). On RTXd56, CGRP(+) IENFs were comparable with the vehicle group ($p = 0.39$). In addition, 4MC normalized thermal analgesia on RTXd35 compared with RTX group ($p = 0.007$). In the current study, the significant promotion of reinnervation of CGRP(+) IENFs and

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thermal latencies on RTXd35 by 4MC indicated that CGRP(+) IENFs were responsible for the thermal transmission in chronic phase of RTX-induced neuropathy.

Introduction

Calcitonin gene-related peptide (CGRP) and substance P (SP) are neurotransmitters that are used by peptidergic small-diameter dorsal root ganglia (DRGs) neurons and are presumably responsible for transmitting nociceptive stimuli from intraepidermal nerve fibers (IENFs) in the skin. These neurons also express transient receptor potential vanilloid subtype 1 (TRPV1) [1,2], which can be depleted by an ultrapotent capsaicin agonist, resiniferatoxin (RTX) [2—4]. RTX is widely used in pain studies to deplete the IENFs, which leads to thermal analgesia [5—8]. Previous studies focused on the depletion of TRPV1(+) neurons and their cutaneous terminal, which corresponds to the thermal transmission [3,4]. Together with the development of thermal analgesia by RTX, those observations raise the following issues: what is the effect of RTX in the depletion of SP(+) and CGRP(+) IENFs and are they in the same degree? If there is a differential effect by RTX, which phenotypic IENFs are susceptible and which profiles of phenotypic IENFs parallel the recovery of thermal sensation in the chronic phase of RTX-induced neuropathy?

4-Methylcatechol (4MC) has been considered as a potent stimulator of endogenous nerve growth factor (NGF) [9—11], which enhanced skin reinnervation [8,11]. In clinics, patients with thermal analgesia are accompanied by skin denervation. These observations implied that the IENFs reinnervation could relieve thermosensation disorders. Our previous study demonstrated that 4MC promoted the synthesis of NGF [11] and skin reinnervation, particularly by CGRP IENFs [8]. Taken together, these results suggest the potential for a therapeutic strategy to improve the skin reinnervation and to reverse the thermal analgesia. The following issues were raised: (1) whether 4MC has the differential effect in peptidergic IENFs, and (2) whether 4MC could promote specific phenotypic IENFs reinnervation that reversed thermal analgesia?

To address these issues, we generated a RTX-induced thermal analgesic mouse model and investigated the depletion of SP(+) and CGRP(+) IENFs with footpad skin sections. After the administration of 4MC, there were significant increases in the densities of CGRP(+) IENFs, while there were reductions in the duration of thermal analgesia, suggesting that CGRP(+) IENFs are responsible for the transmission of thermal nociception.

Materials and methods

RTX-induced neuropathy and 4MC treatment

Experiments were performed on 8-week-old adult male imprinting control region mice (35—40 g). RTX (Sigma, St. Louis, MO, USA) was dissolved in a vehicle (10% Tween-80 and 10% ethanol in normal saline) [2,8]. The animals received a single dose of RTX by intraperitoneal injection (50 μg/kg, defined as the RTX group). One group received an equal volume of vehicle to serve as the controls (the vehicle group). 4MC (10 μg/kg; Wako, Osaka, Japan) was dissolved in phosphate-buffered saline and was intraperitoneally injected to mice on day 7 of RTX-induced neuropathy (RTXd7). The group that received the 4MC injections was defined as the 4MC group [11]. After treatment, mice were housed in plastic cages on 12-hour light/12-hour dark cycle and had access to water and food *ad libitum*. All procedures were conducted in accordance with the ethical guidelines for laboratory animals [12] and the protocol (Permit No. 100055) was approved by Kaohsiung Medical University, Kaohsiung, Taiwan. All experimental procedures were performed using 4% chloral hydrate (dose: 1 mL/100 g), and all efforts were made to minimize suffering.

Evaluation of hot plate withdrawal latencies

Mice were placed on a 52 °C hot plate (IITC, Woodland Hills, CA, USA) enclosed in a Plexiglass cage. The withdrawal latencies of the hind paw to thermal stimulations were determined to an accuracy of 0.1 seconds. Each test session consisted of three trials separated by 30-minute intervals. The criteria of withdrawal included shaking, licking, or jumping on the hot plate. The mean latency was expressed as the threshold of an individual animal to thermal stimulation.

Immunohistochemistry of footpad

Mice were killed by intracardiac perfusion with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (4P) in 0.1 M PB (pH = 7.4) [13]. Footpad tissues were removed after perfusion and postfixed in 4P overnight, followed by cryoprotection with 30% sucrose. Tissues sections, 30-μm thick, were cut on a sliding microtome. Briefly, the sections were quenched with 1% H_2O_2 in methanol and blocked with 0.5% nonfat dry milk and 0.1% Triton X-100 in 0.5 M Tris buffer (Tris). The tissue sections were incubated with primary anti-SP (1:1000; DiaSorin, Stillwater, MN, USA) and CGRP (1:1000; Sigma) antibodies overnight at 4 °C, followed by incubation with biotinylated secondary antibody for another 1 hour. Then the avidin—biotin complex (Vector Labs, Burlingame, CA, USA) method was used and the activity of the reaction product was demonstrated with 3,3′-diaminobenzidine (Sigma). The sections were mounted on gelatin-coated slides for quantification.

Quantification of SP(+) and CGRP(+) IENFs

SP(+) and CGRP(+) IENFs were counted under 400× magnification (Axiophot microscope; Zeiss, Oberkochen,