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Full Length Article

Zinc deficiency promotes cystitis-related bladder pain by enhancing function and expression of $Ca_v3.2$ in mice

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ABSTRACT

$Ca_v3.2$ T-type Ca^{2+} channel activity is suppressed by zinc that binds to the extracellular histidine-191 of $Ca_v3.2$, and enhanced by H_2S that interacts with zinc. $Ca_v3.2$ in nociceptors is upregulated in an activity-dependent manner. The enhanced $Ca_v3.2$ activity by H_2S formed by the upregulated cystathionine-γ-lyase (CSE) is involved in the cyclophosphamide (CPA)-induced cystitis-related bladder pain in mice. We thus asked if zinc deficiency affects the cystitis-related bladder pain in mice by altering $Ca_v3.2$ function and/or expression. Dietary zinc deficiency for 2 weeks greatly decreased zinc concentrations in the plasma but not bladder tissue, and enhanced the bladder pain/referred hyperalgesia (BP/RH) following CPA at 200 mg/kg, a subeffective dose, but not 400 mg/kg, a maximal dose, an effect abolished by pharmacological blockade or gene silencing of $Ca_v3.2$. Acute zinc deficiency caused by systemic *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)-ethylendiamine (TPEN), a zinc chelator, mimicked the dietary zinc deficiency-induced $Ca_v3.2$-dependent promotion of BP/RH following CPA at 200 mg/kg. CPA at 400 mg/kg alone or TPEN plus CPA at 200 mg/kg caused $Ca_v3.2$ overexpression accompanied by upregulation of Egr-1 and USP5, known to promote transcriptional expression and reduce proteasomal degradation of $Ca_v3.2$, respectively, in the dorsal root ganglia (DRG). The CSE inhibitor, β-cyano-L-alanine, prevented the BP/RH and upregulation of $Ca_v3.2$, Egr-1 and USP5 in DRG following TPEN plus CPA at 200 mg/kg. Together, zinc deficiency promotes bladder pain accompanying CPA-induced cystitis by enhancing function and expression of $Ca_v3.2$ in nociceptors, suggesting a novel therapeutic avenue for treatment of bladder pain, such as zinc supplementation.

1. Introduction

$Ca_v3.2$ T-type Ca^{2+} channels are expressed in the primary afferents and play an important role in pain processing (Sekiguchi and Kawabata, 2013; Zamponi et al., 2015). $Ca_v3.2$ is now considered a therapeutic target for treatment of neuropathic and visceral pain (Sekiguchi et al., 2016) including colonic (Matsunami et al., 2009; Tsubota-Matsunami et al., 2012), pancreatic (Nishimura et al., 2009; Terada et al., 2015) and bladder pain (Matsunami et al., 2012). The function of $Ca_v3.2$ is regulated by a number of extracellular substances; e.g. the channel activity is enhanced by extracellular L-cysteine and hydrogen sulfide (H_2S), and suppressed by zinc and ascorbate (Sekiguchi and Kawabata, 2013; Zamponi et al., 2015). Actually, supplementation of zinc or ascorbate attenuates neuropathic or visceral pain in distinct animal models, most probably by inhibition of $Ca_v3.2$ channels (Matsunami et al., 2011; Murakami-Nakayama et al., 2015; Okubo et al., 2012b). The transcriptional expression of $Ca_v3.2$ is positively and negatively regulated by early growth response-1 (Egr-1) and repressor element-1 silencing transcription factor (REST), respectively (Fukami et al., 2015; van Loo et al., 2012), and the proteasomal degradation of $Ca_v3.2$ is suppressed by ubiquitin-specific protease 5 (USP5) (Garcia-Caballero et al., 2014). The upregulation of Egr-1 and/or USP5 in the sensory neurons contributes to the overexpression of $Ca_v3.2$ in animal models for neuropathic pain (Garcia-Caballero et al., 2014; Tomita et al., 2015). Most interestingly, $Ca_v3.2$ in nociceptors can be upregulated following persistent neuronal excitation, which may involve the neuronal activity-induced USP5 upregulation (Stemkowski et al., 2016).

Zinc is one of the essential trace elements, and regulates the function of various proteins including metalloproteins, thereby playing critical roles for a lot of biological functions like acquisition of taste and

Abbreviations: AS, distinct antisense; CPA, cyclophosphamide; CSE, cystathionine-γ-lyase; DRG, dorsal root ganglia; Egr-1, early growth response-1; ODN, oligodeoxynucleotide; REST, repressor element-1 silencing transcription factor; SC-ODN, control scrambled ODN; TPEN, *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)-ethylendiamine; USP5, ubiquitin-specific protease 5

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olfaction, skin metabolism and defense mechanism (Fukada et al., 2011; Murakami and Hirano, 2008; Rink and Haase, 2007). In physiological conditions, interestingly, zinc inhibits the channel activity of $Ca_v3.2$ by binding to a histidine residue at a position 191 (His191) present in the channel's extracellular domain (Sekiguchi and Kawabata, 2013; Zamponi et al., 2015). Zinc chelators thus cancel the zinc inhibition of the $Ca_v3.2$ channel activity, leading to somatic and colonic pain sensitivity (Matsunami et al., 2011; Nelson et al., 2007). Interestingly, like zinc chelators, L-cysteine and H_2S, which are also very sensitive to zinc, interact with zinc binding to His191 of $Ca_v3.2$ and enhance the channel activity by reducing the zinc inhibition, thereby enhancing the excitability of nociceptors (Maeda et al., 2009; Nelson et al., 2007; Okubo et al., 2012a; Sekiguchi and Kawabata, 2013).

In a cyclophosphamide (CPA)-induced cystitis mouse model, the bladder pain is abolished by pharmacological inhibition or gene silencing of $Ca_v3.2$ (Matsunami et al., 2012) and by an inhibitor of cystathionine-γ-lyase (CSE), an H_2S-generating enzyme. It is noteworthy that CSE protein is overexpressed in the bladder tissue after CPA treatment (Matsunami et al., 2012), suggesting a pronociceptive role of the CSE/H_2S/$Ca_v3.2$ pathway in the cystitis-related bladder pain signaling. Most interestingly, polaprezinc, a zinc donor, attenuates the $Ca_v3.2$-dependent bladder pain in the mice with CPA-induced cystitis (Murakami-Nakayama et al., 2015). Collectively, it is hypothesized that extracellular zinc concentrations might determine the channel activity of $Ca_v3.2$, thereby controlling bladder pain sensitivity in mice with cystitis. To address this hypothesis, we thus examined the effect of zinc deficiency on the bladder pain in the mice with CPA-induced cystitis and analyzed possible involvement of $Ca_v3.2$.

2. Methods

2.1. Experimental animals

In the present study, female mice were used, considering the clinical evidence that women show higher incidence of bladder disorders and severer pelvic pain than men (Yosef et al., 2016). Female ddY mice (18–25 g) were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan), and housed in a temperature-controlled room under a 12-h day/night cycle at about 24 °C with free access to food and water. The animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kindai University, and the animal use procedures employed in this study were in accordance with the NIH guidelines (Guide for Care and Use of Laboratory Animals, NIH Publication 86-23).

2.2. Creation of zinc deficient mice by dietary zinc deficiency or by administration of a zinc chelator

To examine the effect of dietary zinc deficiency, the mice were fed with the standard (control) diet containing 48.9 mg zinc/kg or with the zinc-deficient (low zinc) diet containing 0.6 mg zinc/kg (Oriental Yeast Co., Ltd., Tokyo, Japan) for two weeks, as reported elsewhere (Takeda et al., 2012), and then received i.p. administration of CPA at a low (subeffective) dose, 200 mg/kg, or at a high (maximal) dose, 400 mg/kg. To test the effect of acute zinc deficiency, *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)-ethylendiamine (TPEN), a zinc chelator, at 5 mg/kg (Zhang et al., 2012; Zhao et al., 2011) was administered i.p. to mice 30 min before i.p. administration of CPA at 200 mg/kg, the subeffective dose.

2.3. Creation of CPA-induced cystitis and evaluation of bladder pain/referred hyperalgesia in mice

CPA-induced cystitis was created in mice, according to the previous reports (Laird et al., 2002; Miki et al., 2011; Olivar and Laird, 1999) with minor modifications. Briefly, the mice received i.p. administration of cyclophosphamide (CPA; Sigma-Aldrich, St. Louis, MO) at two different doses, 400 mg/kg (maximal dose) and 200 mg/kg (subeffective dose). Bladder pain-like nociceptive behavior was observed from 3.5–4 h after the CPA treatment, immediately followed by evaluation of referred hyperalgesia by von Frey test. For the behavioral observation and nociception test, each mouse was placed on raised wire-mesh floor under a clear transparent plastic box (23.5 × 16.6 × 12.4 cm), and acclimated to the environment at least for 1 h. Bladder pain-like nociceptive behavior, such as licking and/or biting of the skin of the lower abdomen close to the bladder and pressing the lower abdomen against the floor, was observed and counted for 30 min starting 3.5 h after i.p. CPA. Then, to test referred hyperalgesia, the region between the anus and urethral opening was stimulated mechanically using four distinct von Frey filaments with strengths of 0.008, 0.07, 0.4 and 1.0 g, in an ascending order of strength. The stimulation with each filament was repeated 10 times at intervals of 5–10 s and the nociceptive responses were scored as follows: score 0 = no response; score 1 = licking or biting of the external urethral opening and/or the surrounding area, leaving the position, bending of the trunk, raising the upper half of the body, and thrashing limbs; score 2 = jumping. The data are expressed as the total score of responses to 10 challenges with each filament. After von Frey test, the mouse was euthanized by cervical dislocation, and the excised bladder was weighed to evaluate the bladder swelling.

2.4. Measurement of zinc concentrations in the plasma and bladder tissue

The heparinized plasma sample and isolated bladder tissue were incubated in 60% nitric acid solution at 90 °C for 2 h and left to stand at room temperature for 24 h. The plasma and tissue zinc concentrations were then determined by inductively coupled plasma (ICP) mass spectrometry (ICPM-8500, Shimazu, Kyoto, Japan).

2.5. Silencing of $Ca_v3.2$ in the sensory nerves of mice

Knockdown of $Ca_v3.2$ in the sensory nerves was achieved by repeated intrathecal (i.t.) administration of the mixture of two distinct antisense (AS) oligodeoxynucleotides (ODNs) for $Ca_v3.2$ (AS-ODN-$Ca_v3.2$). The AS-ODN-$Ca_v3.2$ and the control scrambled ODNs (SC-ODN) were synthesized by Sigma-Aldrich Japan (Ishikari, Japan). The sequences of the ODNs were: TGA AGT GGT AAT GGT GGT GAT GGT GGT (No. 1) and GAG TGA TGA TGG ACA GGA ACG AGA CCG (No. 2) (AS-ODN-$Ca_v3.2$); TAA GTG GTA TGA GGG TGT TTG GGA (No. 1) and GGG AAA GAC CAC GGG TAA TGG TAG GAC (No. 2) (SC-ODN). A mixture of two types of AS-ODN-$Ca_v3.2$ or SC-ODN (each 2 μg/μL) in a volume of 5 μL was administered i.t. repeatedly, once a day for 3 days in mice.

2.6. Western blot analysis of protein expression

The dorsal root ganglia (DRG) at L1–L6 levels and bladder were excised and frozen in liquid nitrogen. The tissue protein levels were determined by Western blotting, as described previously (Matsunami et al., 2012). Briefly, the tissue samples were homogenized and sonicated in RIPA buffer [PBS, 1% Igepal CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate and 0.1% SDS] containing 0.1 mg/mL phenylmethylsulfonylfluoride, 0.15 U/mL aprotinin and 1 mM sodium orthovanadate. The supernatant, after addition of 2-mercaptoethanol and bromophenol blue, was incubated at 95–100 °C for 5 min. The denatured proteins were separated by electrophoresis on 7.5% (for detection of $Ca_v3.2$, Egr-1, USP-5, REST) or 12.5% (for detection of CSE, GAPDH) SDS-polyacrylamide gels (Wako Pure Chem., Osaka, Japan) and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Billerica, MA). The membrane was blocked with a blocking solution containing 5% skim milk (137 mM NaCl, 0.1% Tween 20 and 20 mM Tris–HCl (pH 7.6)). After washing, the membrane was incubated overnight at 4 °C with the rabbit polyclonal antibodies