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Sensitization of sodium channels by cystathionine β-synthetase activation in colon sensory neurons in adult rats with neonatal maternal deprivation

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ABSTRACT

Background: The pathogenesis of pain in irritable bowel syndrome (IBS) is poorly understood and treatment remains difficult. We have previously reported that TTX-resistant (TTX-R) sodium channels in colon-specific dorsal root ganglion (DRG) neurons were sensitized and the expression of the endogenous hydrogen sulfide producing enzyme cystathionine β-synthetase (CBS) was upregulated in a rat model of visceral hypersensitivity induced by neonatal maternal deprivation (NMD). However, the detailed molecular mechanism for activation of sodium channels remains unknown. This study was designed to examine roles for CBS–H_2S signaling in sensitization of sodium channels in a previously validated rat model of IBS.

Methods: Neonatal male rats (postnatal days 2–15) were exposed to a 3 hour period of daily maternal separation with temperature maintained at ~33 °C. Colon-specific dorsal root ganglion (DRG) neurons were labeled with DiI and acutely dissociated for measuring excitability and sodium channel current under whole-cell patch clamp configurations. The expression of $Na_V1.8$ was analyzed by Western blot and Immunofluorescence study. The endogenous H_2S producing enzyme CBS antagonist was injected intraperitoneally.

Results: We showed that CBS was colocalized with $Na_V1.8$ in colon-specific DRG neurons pre-labeled with DiI. Pretreatment of *O*-(Carboxymethyl) hydroxylamine hemihydrochloride (AOAA), an inhibitor of CBS, significantly reduced expression of $Na_V1.8$ in NMD rats. AOAA treatment also inhibited the TTX-R sodium current density, right-shifted the $V_{1/2}$ of activation curve, and reversed hyperexcitability of colon-specific DRG neurons in NMD rats. Conversely, addition of NaHS, a donor of H_2S, greatly enhanced TTX-R sodium current density, left shifted the activation curve and enhanced excitability of colon DRG neurons in age-matched healthy rats. Furthermore, application of H-89, an inhibitor of protein kinase A, markedly attenuated the potentiation of TTX-R sodium current density by NaHS.

Conclusion: These data suggest that sensitization of sodium channels of colon DRG neurons in NMD rats is most likely mediated by CBS–H_2S signaling, thus identifying a potential target for treatment for chronic visceral pain in patients with IBS.

Introduction

Irritable bowel syndrome (IBS) remains a common and challenging disorder for clinicians. It is defined by recurrent symptoms of abdominal pain or discomfort associated with alterations in bowel habits. The pathophysiology of pain in IBS involves psychological disorder, altered intestinal motility and visceral hypersensitivity (Drossman et al., 2002; Sandler et al., 1984). However, the exact causes of IBS have not been clearly elucidated and effective therapeutics for the primary symptoms has been unavailable. Recent studies in rodents found that early life trauma in the form of neonatal maternal deprivation (NMD) induced rats to develop visceral hypersensitivity at adult, mimicking main pathophysiological features of IBS in human (Hu et al., 2013; Li et al., 2012; Luo et al., 2011). Indeed, early traumatic experiences such as acute bacterial gastroenteritis or childhood abuse situations have been shown to increase the risk of IBS development (Bradford et al., 2012; Heitkemper et al., 2011; Thabane et al., 2010). The visceral hypersensitivity is distinct from those of inflammatory pain and neuropathic pain in that it produces visceral hyperalgesia without involving inflammatory responses in the gut mucosa; the latter is characteristic of IBS. Therefore, NMD in rats have been used as an animal model to study the mechanisms of IBS (Hu et al., 2013; Li et al., 2012; Luo et al., 2011).

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Evidence showed that NMD involves an increase in excitability of primary afferent nociceptors, which convey peripheral stimuli into action potentials that propagate to the central nervous system (Li et al., 2012; Luo et al., 2011). Sensitization of primary sensory neurons is maintained by a number of ion channels such as transient receptor potential channels (Winston et al., 2007), P2X3 receptors (Xu and Huang, 2002), and voltage-gated sodium (VGSCs), potassium (VGPCs) and calcium channels (VGCCs) (Beyak and Vanner, 2005; Hu et al., 2013; Luo et al., 2011; Yoshimura et al., 2001). Voltage-gated sodium channels (VGSCs) are integral membrane glycol-proteins that are essential for generation and conduction of electrical impulses in excitable cells, thus playing a fundamental role in controlling neuronal excitability. The increase in sodium currents density may contribute to the enhanced neuronal excitability. Molecularly, the subunits of mammalian sodium channels have been categorized into nine different subtypes ($Na_V1.1$–$Na_V1.9$). Pharmacologically, sodium channels have been classified according to their sensitivity to the blocker tetrodotoxin (TTX) wherein the currents conducted by $Na_V1.1$–1.4, 1.6, and 1.7 are completely blocked, whereas the currents carried by $Na_V1.5$, $Na_V1.8$, and $Na_V1.9$ are resistant or insensitive to TTX. Since dorsal root ganglion (DRG) neurons do not express $Na_V1.5$, we therefore used TTX to determine roles for $Na_V1.8$ and $Na_V1.9$ in potentiation of total sodium currents after NMD treatment. We have previously reported that VGSCs plays a role in NMD-induced visceral pain (Hu et al., 2013). However, the detailed mechanism underlying the sensitization of VGSCs remains unknown under this condition. Recently, we showed that the endogenous hydrogen sulfide (H_2S) producing enzyme cystathionine β-synthetase (CBS) was upregulated after NMD treatment (Li et al., 2012; Luo et al., 2011). In addition, H_2S has been reported to enhance the sodium current density of DRG neurons from healthy rats (Qi et al., 2013). Therefore, we hypothesize that upregulation of CBS expression contributes to sensitization of VGSCs in colon specific DRG neurons, thus leading to chronic visceral hypersensitivity. As part of an ongoing investigation, we focused on roles of CBS–H_2S signaling in modulating expression and function of VGSCs of colon DRG neurons. We showed that CBS inhibitor significantly suppressed $Na_V1.8$ expression, reduced TTX-R current densities, and lowered neuronal excitability in NMD rats. Conversely, NaHS enhanced TTX-R sodium current densities and excitability of colon specific DRG neurons from healthy rats. Our findings implicate an important role for CBS–H_2S signaling in modulation of sodium channel activities in a rat model of IBS-like visceral hyperalgesia and identify the CBS as a potential molecular target for the treatment of visceral pain under this condition.

Materials and methods

Induction of chronic visceral hyperalgesia (CVH)

Experiments were performed on male Sprague–Dawley rats. Care and handling of these animals were approved by the Institutional Animal Care and Use Committee of Soochow University and were in accordance with the guidelines of the International Association for the Study of Pain. The CVH was induced by neonatal maternal deprivation (NMD), as described previously (Hu et al., 2013; Li et al., 2012). In brief, pups for the NMD group were separated from the maternity cage and placed in isolated cages, using an electric blanket to keep them warm (32 °C) for 3 h daily from postnatal days (PND) 2 to 15. After the separation period, pups were returned to their maternity cage. Pups for the control (CON) group were not exposed to handling and were maintained in their maternity cage with the dam. On PND 21–22, the sex of pups from both groups was determined. Female pups were culled, and male pups were weaned and housed in individual cages in the same way. When rats were 6 weeks old, DiI was injected into colon wall (see description below). These rats were divided into two groups. The first group of rats was intraperitoneally injected once per day with CBS inhibitor, *O*-(Carboxymethyl) hydroxylamine hemihydrochloride (AOAA) and the second group with normal saline (NS). After 7 days of injection, tissues from these animals were used either for patch clamp recordings or for Western blot analysis. A total of 39 rats (CON: n = 4 for excitability recordings, n = 8 for Na^+ current recordings, n = 4 for Western blot analysis, n = 3 for Immunofluorescence; NMD: n = 6 for excitability recordings, n = 4 for Na^+ current recordings, n = 10 for Western blot analysis) were used in this experiments.

Cell labeling

Colon-specific DRG neurons were labeled by injection of 1, 1′-dioleyl-3, 3, 3′, 3-tetramethylindocarbocyanine methanesulfonate (DiI, Invitrogen Corporation) into the colon wall (Hu et al., 2013; Li et al., 2012). Briefly, when the rats were 6 weeks old, they were anesthetized with ketamine (80 mg/kg, i. p.) plus xylazine (5–10 mg/kg, i. p.). The abdomen was opened by midline laparotomy and the colon was exposed. DiI, was injected in ~1 μl volume (25 mg in 0.5 ml methanol) at 10 sites on the exposed colon extending from the level of the bladder to ~6 cm in an oral direction. To prevent leakage and possible contamination of adjacent organs with the dye, the needle was left in place for 1 min and each injection site was washed with normal saline (NS) following each injection. The colon was gently swabbed prior to closing of the abdomen. Rats were returned to their housing and given free access to drinking water and standard food pellets.

Dissociation of DRG neurons and patch clamp recording

Ten days after DiI injection, NMD (~7 weeks) or age-matched control rats were killed by cervical dislocation, followed by decapitation (Hu et al., 2013; Li et al., 2012; Zhang et al., 2013). DRGs (T_{13}–L_2) were bilaterally dissected out and transferred to an ice-cold, oxygenated fresh dissecting solution, containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgSO_4$, 10 glucose and 10 HEPES, pH 7.2 (osmolarity: 305 mOsm). After removal of the connective tissue, the ganglia were transferred to a 5 ml dissecting solution containing collagenase D (1.8-2.0 mg/ml; Roche Diagnostics, Indianapolis, Indiana, USA) and trypsin (1.2–1.5 mg/ml; Sigma-Aldrich Corporation, St Louis, Missouri, USA) and incubated for 1.5 h at 34.5 °C. DRGs were taken from the enzyme solution, washed and transferred to 0.5 ml of the dissecting solution containing DNase (0.5 mg/ml; Sigma-Aldrich Corporation, St Louis, Missouri, USA). A single cell suspension was subsequently obtained by repeated trituration through flame-polished glass pipettes. Cells were plated onto acid-cleaned glass coverslips. Coverslips containing adherent DRG cells were put in a small recording chamber (1 ml volume) and attached to the stage of an inverted microscope (Olympus IX71) fitted for both fluorescence and bright-field microscopy. DiI-labeled neurons were identified by their fluorescence under the fluorescent microscope. For the patch-clamp recording experiments, cells were continuously superfused (1.5 ml/min) at room temperature with normal external solution containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with pH adjusted to 7.2 with NaOH, osmolarity: 295–300 mOsm. Recording pipettes were pulled from borosilicate glass tubing using a horizontal puller (P-97, Sutter Instruments). Unless indicated, patch-clamp pipettes had a resistance of 4–7 MΩ when filled with the pipette solution containing (in mM): 140 potassium gluconate, 10 NaCl, 10 HEPES, 10 glucose, 5 EGTA and 1 $CaCl_2$, pH = 7.25 adjusted with KOH; osmolarity: 292 mOsm. Under current clamp condition, resting potential (RP) and action potentials (APs) evoked by 2 and 3 times rheobase current stimulation and by ramp current stimulation were recorded by a HEKA EPC10 patch clamp amplifier (HEKA Electronik GmBH; Germany). Capacitive transients were corrected using capacitive cancelation circuitry on the amplifier that yielded the whole cell capacitance and access resistance. Up to 90% of the series resistance was compensated electronically. Considering the peak outward current amplitudes of <10 nA, the estimated voltage errors from the uncompensated series resistance would be