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RESEARCH PAPER

Hydrogen sulfide-induced mechanical hyperalgesia and allodynia require activation of both $Ca_V3.2$ and TRPA1 channels in mice

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BACKGROUND AND PURPOSE

Hydrogen sulfide, a gasotransmitter, facilitates somatic pain signals via activation of $Ca_V3.2$ T-type calcium channels in rats. Given evidence for the activation of transient receptor potential ankyrin-1 (TRPA1) channels by H_2S, we asked whether TRPA1 channels, in addition to $Ca_V3.2$ channels, contribute to the H_2S-induced mechanical hyperalgesia and allodynia in mice.

EXPERIMENTAL APPROACH

Mechanical hyperalgesia and allodynia were evaluated by the von Frey test in mice. $Ca_V3.2$ or TRPA1 channels in the sensory neurons were silenced by repeated intrathecal administration of antisense oligodeoxynucleotides in mice.

KEY RESULTS

Intraplantar administration of NaHS evoked hyperalgesia and allodynia in mice, an effect attenuated or abolished by NNC 55–0396 or mibefradil, T-type calcium channel blockers, and by ascorbic acid or zinc chloride, known to selectively inhibit $Ca_V3.2$ channels, out of the three isoforms of T-type calcium channels. Silencing of $Ca_V3.2$ channels in the sensory neurons also prevented the NaHS-induced hyperalgesia and allodynia in mice. The NaHS-induced hyperalgesia and allodynia in mice were significantly suppressed by AP18, a TRPA1 channel blocker, and by silencing of TRPA1 channels in the sensory neurons.

CONCLUSIONS AND IMPLICATIONS

Mechanical hyperalgesia and allodynia induced by NaHS/H_2S required activation of both $Ca_V3.2$ and TRPA1 channels in mice.

Abbreviations

AITC, allyl isothiocyanate; AS, antisense; CSE, cystathionine-γ-lyase; DRG, dorsal root ganglion; i.pl., intraplantar; ODN, oligodeoxynucleotide; TRPA1, transient receptor potential ankyrin-1

Introduction

Hydrogen sulfide, a gasotransmitter, is formed endogenously from L-cysteine mainly by cystathionine-γ-lyase (CSE), cystathionine-β-synthase or cysteine aminotransferase plus 3-mercaptopyruvate sulphurtransferase (Li and Moore, 2008; Shibuya *et al.*, 2009). Various roles of H_2S in health and disease have been reported throughout the mammalian body including neuronal systems over the last decade (Li and Moore, 2008; Kimura, 2010). We have shown that NaHS, a donor of H_2S, accelerates T-type calcium channel currents in NG108-15 cells that abundantly express the $Ca_V3.2$ isoform of T-type calcium channels (channel nomenclature follows Alexander *et al.*, 2011) or in mouse dorsal root ganglion

(DRG) neurons (Matsunami *et al.*, 2009; Nagasawa *et al.*, 2009; Tarui *et al.*, 2010). Activation or sensitization of T-type calcium channels by NaHS causes neurite outgrowth and/or neuronal differentiation in NG108-15 cells (Nagasawa *et al.*, 2009; Tarui *et al.*, 2010), and mechanical hyperalgesia in rats *in vivo* (Kawabata *et al.*, 2007). $Ca_v3.2$ channels are considered to mediate NaHS-induced facilitation of nociceptive processing in rats (Maeda *et al.*, 2009). The activation of $Ca_v3.2$ channels by CSE-derived H_2S is involved in the experimental neuropathic pain caused by L5 spinal nerve injury or by repeated administration of paclitaxel, an anti-cancer drug, in rats (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). There is also evidence that the $H_2S/Ca_v3.2$ channel pathway contributes to processing of visceral nociception including colonic and pancreatic pain in mice (Matsunami *et al.*, 2009; Nishimura *et al.*, 2009).

Transient receptor potential ankyrin-1 (TRPA1) channels are abundantly expressed in sensory neurons, and function to sense noxious cold (<17°C) and some other nociceptive stimuli (Stucky *et al.*, 2009). Most interestingly, it has also been reported that NaHS increases cytosolic calcium concentrations in TRPA1-transfected cells (Streng *et al.*, 2008), and that NaHS induces calcium influx and inward currents in rat DRG cells, which are inhibited by a TRPA1 antagonist (Miyamoto *et al.*, 2011). These findings suggest that H_2S might activate TRPA1 channels in addition to $Ca_v3.2$ channels in sensory neurons, contributing to the development of H_2S-induced hyperalgesia.

In the present study, we first examined if intraplantar (i.pl.) administration of NaHS caused mechanical hyperalgesia and/or allodynia in mice, as it did in rats, and then asked if TRPA1 channels and/or $Ca_v3.2$ T-type calcium channels contributed to the hyperalgesia and allodynia induced by i.pl. administration of NaHS in mice.

Methods

Animals

All animal care and experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University. Male ddY mice weighing about 18 g were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan) and used for *in vivo* experiments at the body weight of 20–30 g. Animals were housed in a temperature- and light-controlled room (around 24°C, 12-h light/dark cycles) and had free access to food and water.

Evaluation of mechanical hyperalgesia and allodynia in mice using the von Frey test

Mice were placed on a raised wire mesh floor, covered with a clear plastic box (23 × 16 × 12 cm), and acclimated to the experimental environment. The mid-plantar surface was stimulated with filaments with strengths of 0.07, 0.16 and 0.4 g in an ascending order of their strength, at intervals 5–10 s, five times for each filament. The behavioural responses to each stimulation were scored as follows: score 0, no response; score 1, lifting of the paw, aversive response or escaping from stimulation; score 2, shaking of the paw, licking of the paw or jumping. The sum of scores in response to five stimuli with each filament was calculated.

Drug administration

NaHS (Kishida Chem. Co., Ltd. Osaka, Japan), a donor of H_2S, was dissolved in saline. Mice received i.pl. injections of NaHS at doses of 10–100 pmol per paw in a volume of 10 μL into the right hindpaw. Allyl isothiocyanate (AITC; Tokyo Chemical Industry Co., Ltd, Tokyo, Japan), a TRPA1 agonist, was dissolved in peanut oil and administered i.pl. in mice at doses of 3.1–31 nmol per paw in a volume of 10 μL. For inhibition experiments, NNC 55–0396 (Sigma-Aldrich, St. Louis, MO, USA) and mibefradil (Sigma-Aldrich), T-type calcium channel blockers, or ascorbic acid (Sigma-Aldrich) and zinc chloride (Kishida Chemical Co., Ltd), known to inhibit $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$ channels (Nelson *et al.*, 2007a,b), were dissolved in saline. AP18 (Enzo Life Sciences, Farmingdale, NY, USA), a TRPA1 antagonist, was dissolved in a solution containing 7.5% DMSO, 92% PBS and 0.5% Tween 80. NNC 55–0396 was administered i.pl. at 1 nmol per paw or i.p. at 35.4 $\mu mol \cdot kg^{-1}$ in mice. Mibefradil was given i.p. at 17.6 $\mu mol \cdot kg^{-1}$ in mice. AP18 was administered i.p. at 47.7 $\mu mol \cdot kg^{-1}$ in mice. Injection volume for i.pl. injection was 5–10 μL in mice.

Silencing of $Ca_v3.2$ or TRPA1 channels in sensory neurons by intrathecal administration of antisense oligodeoxynucleotides in mice

Knockdown of $Ca_v3.2$ or TRPA1 channels in sensory neurons were achieved by repeated intrathecal administration of antisense (AS) oligodeoxynucleotides (ODNs). Two distinct AS-ODNs targeting mouse $Ca_v3.2$ and scrambled ODNs were synthesized by Sigma-Aldrich Japan (Ishikari, Japan), and the two distinct AS-ODNs or scrambled ODNs were mixed immediately before intrathecal administration. The sequences were as follows: AS-ODNs-$Ca_v3.2$, TGAAGTGGTAATGGTGG TGATGGTGGT and GAGTGATGATGGACAGGAACGAGAC CG; scrambled ODNs-$Ca_v3.2$, TAAGTGGTGGTATGAGGG TGTTTGGGA and GGGAAAGACCACGGGTAATGGTAGGAC. The AS-ODN targeting mouse TRPA1 channels and mismatch ODN were synthesized by Sigma-Aldrich Japan, and the sequences were as follows: AS-ODN-TRPA1, TCTATGCGG TTATGTTGG; mismatch ODN, ACTACTACACTAGACTAC (Andrade *et al.*, 2008). For knockdown of $Ca_v3.2$ channels, the two AS-ODNs-$Ca_v3.2$ or scrambled ODNs (control) at a dose of 1.2 nmol per mouse in a volume of 5 μL were administered intrathecally, once a day for 3 days. For knockdown of TRPA1 channels, the AS-ODN-TRPA1 or mismatch ODN (control) at a dose of 2.5 nmol per mouse in a volume of 5 μL was administered intrathecally, once a day for 3 days.

Western blotting

Silencing of $Ca_v3.2$ or TRPA1 channels in sensory neurons by AS-ODN(s) was confirmed by Western blot analysis of those proteins in mouse DRG. The mice were killed by cervical dislocation. The bilateral DRG at L1–L6 levels were excised from the mice. Each sample was homogenized and sonicated in a RIPA buffer [PBS, 1% Igepal CA-630 (Sigma-Aldrich),

0.5% sodium deoxycholate and 0.1% SDS] containing 0.1 mg·mL^{-1} phenylmethylsulfonylfluoride, 0.15 U·mL^{-1} aprotinin and 1 mM sodium orthovanadate. After centrifugation, glycerol, 2-mercaptoethanol, 10% SDS, 1 M Tris–HCl (pH 6.7) and bromophenol blue were added to the supernatant. Proteins in the sample were denatured at 95–100°C for 5 min and separated by electrophoresis on 7.5% and 12.5% SDS-polyacrylamide gels (Wako Pure Chem., Osaka, Japan) for detection of $Ca_v3.2$ channels and of TRPA1 channels and GAPDH, respectively, and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Billerica, MA, USA). 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 with the affinity-purified anti-$Ca_v3.2$ channel rabbit polyclonal antibody (1:1000 dilution) (Sigma-Aldrich), the anti-TRPA1 channel rabbit polyclonal antibody (1:500 dilution) (Novus Biologicals, Littleton, CO, USA) or the anti-GAPDH rabbit polyclonal antibody (1:5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing, the membrane was incubated with a secondary antibody, the HRP-conjugated anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA). Positive bands for $Ca_v3.2$, TRPA1 channels and GAPDH were identified around 230, 110 and 37 kDa, respectively, by enhanced chemiluminescence staining (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistics

Data are shown as mean ± SEM. Kruskal–Wallis *H*-test followed by a least significant difference-type test was used for non-parametric analyses of the statistical difference among three or more group data, obtained from the experiments employing the von Frey test. Student's *t*-test was used for parametric analyses of the two-group data. Differences among experimental group means were considered significant when $P < 0.05$.

Results

Intraplantar administration of NaHS causes T-type calcium channel-dependent mechanical hyperalgesia and allodynia in mice

Behavioural responses to mechanical stimulation with von Frey filaments were scored to detect both mechanical hyperalgesia and allodynia; 0.07 g filaments and 0.16 g or 0.4 g filaments were used for evaluation of allodynia and hyperalgesia, respectively. Intraplantar administration of NaHS at 10–100 pmol per paw produced significant increases in behavioural scores in response to stimuli with 0.16 g or 0.4 g filaments and with 0.07 g filaments in a dose-dependent manner, indicating the development of mechanical hyperalgesia and allodynia, respectively (Figure 1A). Two pan-T-type calcium channel blockers, mibefradil at 17.6 µmol·kg^{-1} and NNC 55–0396 at 35.4 µmol·kg^{-1}, abolished the hyperalgesia and allodynia caused by i.pl. NaHS (Figure 1B, C). We then asked if ascorbic acid or zinc chloride, known to selectively inhibit the $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$, isoforms of T-type calcium channels (Nelson *et al.*, 2007a,b; Matsunami

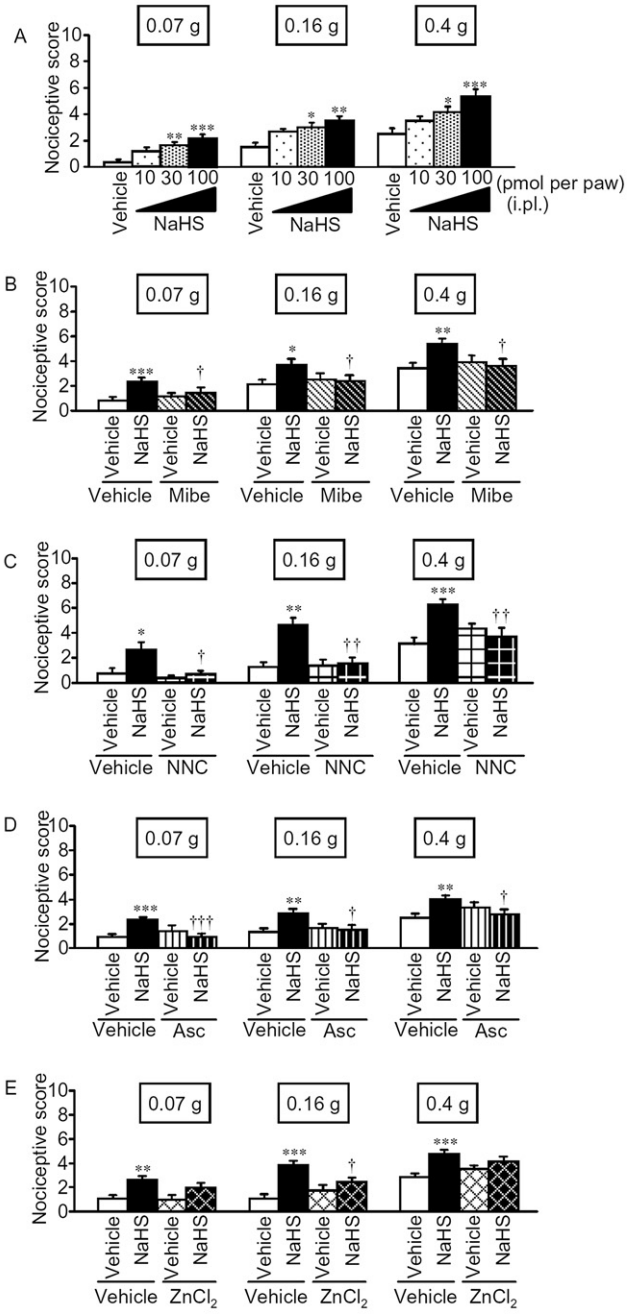

Figure 1

The hyperalgesia and allodynia induced by i.pl. administration of NaHS in mice, and the inhibitory effects of T-type calcium channel blockers. The hyperalgesia and allodynia were evaluated by determining nociceptive scores in response to stimuli with distinct von Frey filaments (0.07, 0.16 or 0.4 g strength). (A) The hyperalgesia/allodynia was evaluated 15–25 min after i.pl. NaHS at 10–100 pmol per paw. (B, C and D) Mibefradil (Mibe) at 17.6 µmol·kg^{-1} (B), NNC 55–0396 (NNC) at 35.4 µmol·kg^{-1} (C) or zinc chloride ($ZnCl_2$) at 2.5 µmol·kg^{-1} (E) was given i.p. 30 min before i.pl. NaHS at 100 pmol per paw. (D) Ascorbic acid (Asc) at 1 nmol per paw was given i.pl. 10 min before i.pl. NaHS. Data show the mean with SEM for six (A), 13–21 (B), 6–9 (C), 12–13 (D) or 8–14 (E) mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle (A) or vehicle plus vehicle (B–E). †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ vs. vehicle plus NaHS.

et al., 2011), affects the pro-nociceptive effects of NaHS. Ascorbic acid, pre-administered i.pl. at 1 nmol per paw, or zinc chloride, pre-administered i.p. at 2.5 μmol·kg^{-1} significantly suppressed the NaHS-induced hyperalgesia and allodynia (Figure 1D, E).

Silencing of $Ca_v3.2$ channels in sensory neurons abolishes the mechanical hyperalgesia and allodynia induced by NaHS in mice

Repeated intrathecal injection of AS-ODNs-$Ca_v3.2$ clearly and significantly suppressed the expression of $Ca_v3.2$ protein in DRG (Figure 2A, B) and strongly inhibited the mechanical hyperalgesia and allodynia evoked by i.pl. NaHS in mice (Figure 2C).

The mechanical hyperalgesia and allodynia induced by i.pl. NaHS are inhibited by a TRPA1 channel antagonist in mice

Intraplantar administration of AITC, a TRPA1 agonist, at 3.1–31 nmol per paw produced mechanical hyperalgesia and allodynia in a dose-dependent manner in mice (Figure 3A). The AITC-evoked hyperalgesia or allodynia was abolished by i.p. pretreatment with AP18, a TRPA1 antagonist, at 47.7 μmol·kg^{-1}, but not by i.pl. injection of NNC 55–0396 at

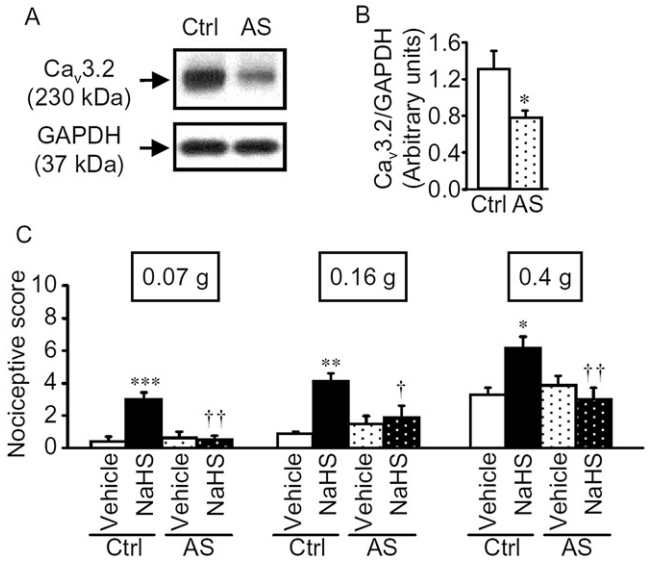

Figure 2

Effect of silencing of $Ca_v3.2$ channels on the allodynia and hyperalgesia induced by i.pl. NaHS in mice. The mice received intrathecal administration of AS-ODNs-$Ca_v3.2$ (AS) or the control ODNs (Ctrl) at a dose of 1.2 nmol per mouse, once a day for 3 days. (A) Representative photographs of Western blots for $Ca_v3.2$ in mouse DRG. (B) The expression levels of $Ca_v3.2$ protein in DRG were quantified by densitometry. (C) The hyperalgesia and allodynia was evaluated 15–25 min after i.pl. NaHS at 100 pmol per paw using distinct von Frey filaments (0.07, 0.16 or 0.4 g strength). Data show the mean with SEM for 7–8 mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Ctrl plus vehicle. †$P < 0.05$, ††$P < 0.01$ vs. Ctrl plus NaHS.

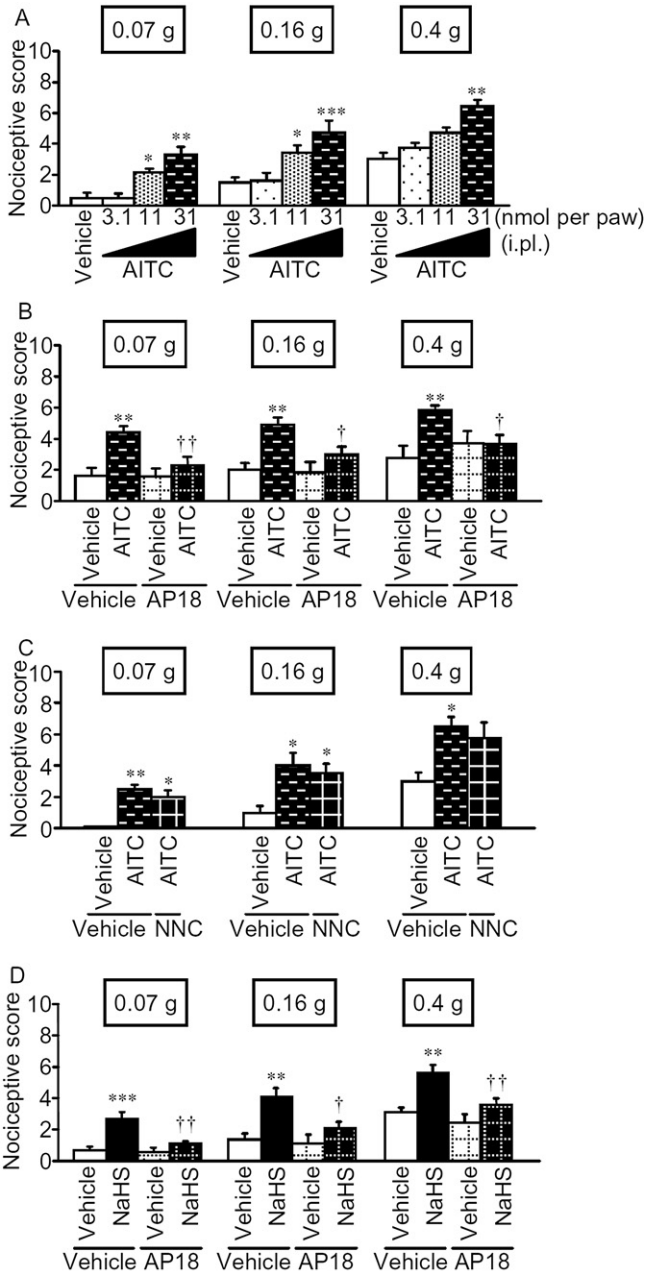

Figure 3

Effects of TRPA1 or T-type calcium channel blockers on the hyperalgesia and allodynia induced by i.pl. administration of AITC, a TRPA1 channel agonist, or NaHS in mice. The hyperalgesia and allodynia were evaluated by determining nociceptive scores in response to stimuli with distinct von Frey filaments (0.07, 0.16 or 0.4 g strength). (A) The hyperalgesia and allodynia was evaluated 15–25 min after i.pl. AITC at 3.1, 11 or 31 nmol per paw. (B, C and D) AP18 at 47.7 μmol·kg^{-1} was given i.p. 30 min before i.pl. AITC at 31 nmol per paw (B) or NaHS at 100 pmol per paw (D), and NNC 55–0396 (NNC) at 1 nmol per paw was given 10 min before i.pl. AITC (C). Data show the mean with SEM for 7–8 (A), 7–11 (B), 4 (C) or 9–15 (D) mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle (A) or vehicle plus vehicle (B-D). †$P < 0.05$, ††$P < 0.01$ vs. vehicle plus AITC (B) or vehicle plus NaHS (D).

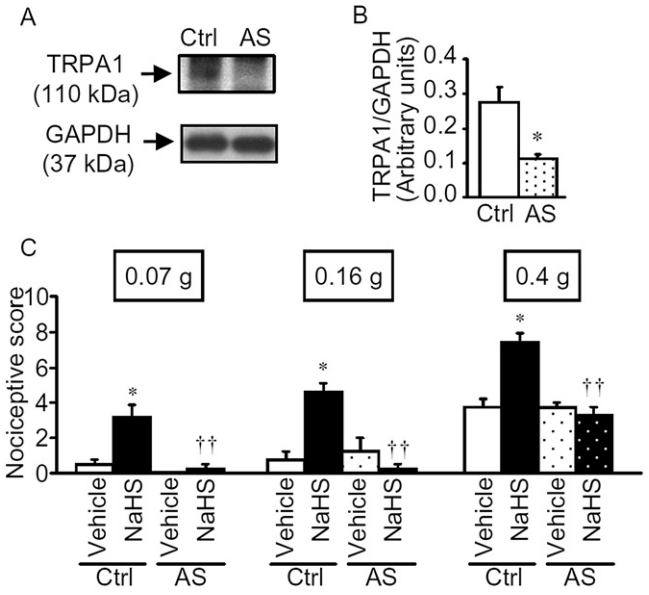

Figure 4

Effect of silencing of TRPA1 channels on the allodynia and hyperalgesia induced by i.pl. NaHS in mice. The mice received intrathecal administration of AS-ODN-TRPA1 (AS) or the control ODNs (Ctrl) at a dose of 2.5 nmol per mouse, once a day for 3 days. (A) Representative photographs of Western blots for TRPA1 in mouse DRG. (B) The expression levels of TRPA1 protein in DRG were quantified by densitometry. (C) The hyperalgesia and allodynia was evaluated 15–25 min after i.pl. NaHS at 100 pmol per paw using distinct von Frey filaments (0.07, 0.16 or 0.4 g strength). Data show the mean with SEM for 4–5 mice. *$P < 0.05$ vs. Ctrl or Ctrl plus vehicle. ††$P < 0.01$ vs. Ctrl plus NaHS.

1 nmol per paw (Figure 3B, C). The same dose of AP18 also inhibited the mechanical hyperalgesia and allodynia induced by i.pl. NaHS (Figure 3D).

Silencing of TRPA1 channels in the sensory neurons abolishes the mechanical hyperalgesia and allodynia induced by NaHS in mice

Repeated intrathecal injection of AS-ODN-TRPA1 greatly and significantly suppressed the expression of TRPA1 protein in DRG (Figure 4A, B), and abolished the mechanical hyperalgesia and allodynia evoked by i.pl. NaHS (Figure 4C).

Discussion

The present results indicate that, as previously shown in rats (Maeda *et al.*, 2009), i.pl. NaHS produced mechanical hyperalgesia and allodynia through the activation of $Ca_v3.2$ T-type calcium channels in mice. Furthermore, our data demonstrated that the i.pl. NaHS-induced mechanical hyperalgesia and allodynia were dependent on TRPA1 channels, in addition to $Ca_v3.2$ channels, in mice.

We have shown that the activation of $Ca_v3.2$ T-type calcium channels by endogenous H_2S formed by CSE or by exogenously applied NaHS plays critical roles in processing of somatic pain signals in naïve rats and in rats with neuropathic pain (Kawabata *et al.*, 2007; Maeda *et al.*, 2009; Takahashi *et al.*, 2010; Okubo *et al.*, 2011). It is particularly of interest that TRPA1 channels in addition to $Ca_v3.2$ T-type calcium channels played crucial roles in the NaHS-induced hyperalgesia and allodynia in mice in the present study. These findings are consistent with the previous *in vitro* evidence that NaHS causes calcium influx in TRPA1-transfected cells, but not in control cells (Streng *et al.*, 2008). There is also *in vitro* evidence that NaHS is capable of activating TRPA1 channels in rat isolated DRG neurons (Miyamoto *et al.*, 2011).

TRPA1 channels are considered to be involved in mechanical nociceptive processing even in naïve mice, considering the present evidence for the AP18-reversible mechanical hyperalgesia and allodynia caused by AITC in mice (see Figure 3) and the previous evidence that TRPA1-null mice exhibit mechanical hypoalgesia (Petrus *et al.*, 2007). In contrast, it is to be noted that stimulation of TRPA1 channels causes peripheral sensitization of heat nociceptors in naïve rats, while not affecting responses to mechanical stimulation (Merrill *et al.*, 2008). Nonetheless, TRPA1 channels in rats appear to be involved in the mechanical hyperalgesia and allodynia in pathological conditions including experimental diabetes (Wei *et al.*, 2010), adjuvant-induced inflammation and spinal nerve injury (Obata *et al.*, 2005). It remains to be investigated whether TRPA1 channels are involved in the NaHS-induced mechanical hyperalgesia or allodynia in rats, although different isoforms or splice variants of TRPA1 channels between rats and mice are not known.

It is to be noted that AS-ODNs for $Ca_v3.2$ or TRPA1 channels abolished the mechanical hyperalgesia or allodynia, although they only partially inhibited the expression levels of target proteins in the homogenates of DRG (see Figures 2 and 4). The following two possibilities might be considered: (i) treatment with the AS-ODNs might strongly reduce the membrane expression of $Ca_v3.2$ or TRPA1 channels, while it might cause relatively minor decrease in the levels of those channels in cytosolic pools; (ii) the extent of suppression of $Ca_v3.2$ or TRPA1 channel expression by the AS-ODNs might be greater in the peripheral ending of the sensory neurons than the cell body present in the DRG. In addition to the involvement of H_2S in processing of somatic pain, exogenously applied NaHS and/or endogenous H_2S formed by CSE causes visceral pain or referred hyperalgesia via $Ca_v3.2$ T-type calcium channels in mice (Matsunami *et al.*, 2009; 2011; Nishimura *et al.*, 2009). Our study is now in progress to ask if TRPA1 channels, in addition to $Ca_v3.2$ channels, contribute to the facilitation of visceral pain signals by H_2S in mice. The activation of TRPA1 channels by AITC appears to result from modification of several intracellular cysteine residues in TRPA1 molecules (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). H_2S is also able to modify the cysteine residue by sulphydration (Sen and Snyder, 2010), which might be involved in the activation of TRPA1 channels. In contrast, the activation of $Ca_v3.2$ channels by H_2S might involve the cancellation of endogenous zinc inhibition of $Ca_v3.2$ channels (Matsunami *et al.*, 2011), which contributes to the activation of $Ca_v3.2$ channels by zinc-chelating agents including L-cysteine (Nelson *et al.*, 2007b).

In conclusion, NaHS/H_2S-induced mechanical hyperalgesia and allodynia required activation of both $Ca_v3.2$ and TRPA1 channels in mice.

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Conflicts of interest

None.

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