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Role of Hydrogen Sulfide in the Development of Colonic Hypomotility in a Diabetic Mouse Model Induced by Streptozocin

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ABSTRACT

Hydrogen sulfide (H_2S), a novel gasotransmitter, is involved in the regulation of gut motility. Alterations in the balance of H_2S play an important role in the pathogenesis of diabetes. This study was conducted to investigate the role of H_2S in the colonic hypomotility of mice with streptozotocin (STZ)-induced diabetes. A single intraperitoneal injection of STZ was used to induce the type 1 diabetes model. Male C57BL/6 mice were randomized into a control group and an STZ-treated group. Immunohistochemistry, Western blotting, H_2S generation, organ bath studies and whole-cell patch clamp techniques were carried out in single smooth muscle cells (SMCs) of the colon. We found that STZ-induced diabetic mice showed decreased stool output, impaired colonic contractility, and increased endogenous generation of H_2S ($p < 0.05$). H_2S-producing enzymes were upregulated in the colon tissues of diabetic mice ($p < 0.05$). The exogenous H_2S donor sodium hydrosulfide (NaHS) elicited a biphasic action on colonic muscle contraction with excitation at lower concentrations and inhibition at higher concentrations. NaHS (0.1 mM) increased the currents of voltage-dependent calcium channels (VDCCs), while NaHS at 0.5 mM and 1.5 mM induced inhibition. Furthermore, NaHS reduced the currents of both voltage-dependent potassium (K_V) channels and large conductance calcium-activated potassium (BK) channels in a dose-dependent manner. These results show that spontaneous contraction of colonic muscle strips from diabetic mice induced by STZ was significantly decreased, which may underlie the constipation associated with diabetes mellitus (DM). H_2S overproduction with subsequent suppression of muscle contraction via VDCCs on SMCs may contribute in part to the pathogenesis of colonic hypomotility in DM.

SIGNIFICANCE STATEMENT

Hydrogen sulfide may exhibit a biphasic effect on colonic motility in mice by regulating the activities of voltage-dependent calcium channels and voltage-dependent and large conductance calcium activated potassium channels. H_2S overproduction with subsequent suppression of muscle contraction via VDCCs may contribute to the pathogenesis of colonic hypomotility in diabetes mellitus.

Introduction

As many as 76% of patients with diabetes mellitus (DM) suffer from digestive system manifestations, such as vomiting, abdominal pain, diarrhea and constipation (Bytzer et al., 2001; Feldman and Schiller, 1983). Among these, constipation caused by impaired colonic contractility is the most common symptom and influences approximately 60% of diabetic patients (Feldman and Schiller, 1983; Maleki et al., 1998). The underlying pathogenesis regarding constipation linked to DM is likely to be multifactorial and complex. Known and emerging mechanisms include autonomic neuropathy, disturbances in the enteric nervous system and interstitial cells of Cajal, as well as defects in gastrointestinal (GI) smooth muscle itself (Yarandi and Srinisavan, 2014; Nakahara et al., 2002; Touw et al., 2012). In addition, endothelial dysfunction and altered microbiota may contribute to DM-induced GI dysmotility (Bódi et al., 2012; Bagyánszki and Bódi, 2012). Nevertheless, the etiology that underlines the decreased colonic motility associated with DM is not fully understood.

Hydrogen sulfide (H_2S) is now increasingly considered a gaseotransmitter and can be endogenously produced by mammalian tissues via cystathionine b-synthase (CBS) and cystathionine c-lyase (CSE) (Kimura, 2011). Both CBS and CSE are expressed along the entire GI tract, including the epithelium, muscle wall, and myenteric plexuses (Jimenez et al., 2017; Gil et al., 2011; Linden et al., 2008). H_2S can travel quickly through cell membranes and exert various physiologic and pathologic functions, causing a variety of biologic responses, including altered gut motility (Jimenez et al., 2017). Many of the biologic responses to H_2S follow a biphasic dose–response (Szabo, 2010). With regard to GI motility, it has been reported

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ABBREVIATIONS: AOAA, amino-oxyacetic acid; BK channel, large conductance calcium activated potassium channel; CBS, cystathionine b-synthase; CM, circular muscle; CSE, cystathionine c-lyase; DM, diabetes mellitus; GI, gastrointestinal; H_2S, hydrogen sulfide; K_V channel, voltage-dependent potassium channel; LM, longitudinal muscle; NaHS, sodium hydrosulfide; SMC, smooth muscle cell; STZ, streptozocin; TTX, tetrodotoxin; VDCC, voltage-dependent calcium channel.

that exogenous H_2S produces contraction at low concentrations and relaxation at high concentrations (Zhao et al., 2009; Han et al., 2011). Furthermore, H_2S induced transient excitatory effects and long-lasting inhibition of muscle contraction in the rat duodenum and colon (Lu et al., 2014). Altered levels of H_2S and expression of H_2S synthase have been frequently reported in diabetic patients and various models of diabetes (Jain et al., 2010; Yusuf et al., 2005; Wu et al., 2009). Increasing evidence shows that H_2S is involved in the development of diabetes and diabetic complications (Gheibi et al., 2020; Szabo, 2012). However, its impact on colonic dysmotility resulting from DM has not been investigated.

Therefore, we hypothesized that H_2S might be involved in colonic motility disorders associated with DM. The current study was conducted to measure the H_2S levels and expression of H_2S-generating enzymes in colons from diabetic mice induced by streptozocin (STZ). The spontaneous contractions of colonic smooth muscle were compared between diabetic mice and healthy controls. Furthermore, the effects of exogenous H_2S on colonic smooth muscle contraction were monitored by organ bath and whole-cell patch clamp recording. Our results demonstrated that H_2S overproduction with subsequent inhibition of smooth muscle contraction may be a causative factor in the pathogenesis of colonic hypomotility in diabetes.

Materials and Methods

Animals. Male C57BL/6 mice (6–8 weeks of age, 20–25 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University and housed under specific pathogen-free conditions (12-hour light/dark cycle, 60% relative humidity, between 22 and 24°C) with free access to food and tap water. All experiments were carried out in accordance with the ethical guidelines of the International Association for the Study of Pain and approved by local ethical review committees. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Xi'an Jiaotong University, Xi'an City, China (Approval ID:2021866).

Chemicals. STZ, sodium hydrosulfide (NaHS), tetrodotoxin (TTX), L-cysteine, L-propargylglycine (PAG) and amino-oxyacetic acid (AOAA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). CBS and CSE polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Hong Kong Ltd., Hong Kong), respectively. STZ was dissolved in citrate buffer, and NaHS, TTX, L-cysteine, L-propargylglycine, and AOAA were dissolved in pure water.

STZ-Induced Type 1 Diabetic Mouse Model. The type 1 diabetes model was induced by a single intraperitoneal injection of STZ (200 mg/kg in ice-cold 0.05 mmol/L citrate buffer). Following an overnight fast, mice were injected intraperitoneally with STZ or vehicle (the same volume of citrate buffer). The level of blood glucose was monitored at three days and two weeks after STZ injection and evaluated again four weeks later. Mice with a blood glucose level above 16.7 mmol/L were considered type 1 diabetes model mice as previously described (Song et al., 2018). The average number of feces per mouse per hour was calculated based on the 24-hour period fecal pellet output after four weeks of STZ injection. All animals were euthanized at four weeks after STZ injection.

Preparation of Muscle Strips and Organ Bath Studies. Mice were intraperitoneally anesthetized with 10% (w/v) chloral hydrate. The middle colon was excised and immediately placed in a plate filled with oxygenated Tyrode's buffer (pH 7.4) of the following composition in mM: NaCl at 147.0, KCl at 4.0, $CaCl_2$ at 2.0, NaH_2PO_4 at 0.42, Na_2HPO_4 at 2.0, $MgCl_2$ at 1.05, and glucose at 5.5. After cutting off the mesenteric fat, the colon was opened along the mesenteric border and fixed with mucosa facing up. The mucosa was then removed by sharp dissection under a microscope. The longitudinal muscle (LM) and circular muscle (CM) strips with submucosal layer (approximately 3 mm×8 mm) were prepared by cutting along the longitudinal and circumferential directions, respectively. Each tissue preparation was suspended in an 8-ml organ bath containing Tyrode's buffer maintained at 37°C and gassed with 95% O_2 and 5% CO_2. The oral end of the preparations was connected to an isometric force transducer (JZJOIH, Chengdu, China), and the anal end was fixed to a hook at the bottom of the bath. The muscle strips were initially loaded to a tension of 1 g, followed by equilibration for 60 minutes, during which time they were washed twice. The spontaneous contractile activities of the strips were recorded and transduced to a computer using an RM6240 multichannel physiologic signaling system (JZJOIH, Chengdu, China).

Endogenous Production of Hydrogen Sulfide. The generation of H_2S was detected as described previously (Gil et al., 2011). Briefly, the full-thickness colon tissues were incubated in a sealed vial containing Tyrode's buffer with L-cysteine (10 mM) and pyridoxal 59-phosphate (2 mM). The vial incubated with tissues was linked to a 2-ml vial containing 0.5 ml of zinc acetate (1%, w/v). The first vial was bubbled with 97% O_2 and 3% CO_2 at a speed of 2 ml/min to inhibit the spontaneous degradation of H_2S. Increased pressure in the first vial forced gas to move through the silastic tube connected to the second vial, and H_2S was then trapped as zinc sulfide. The reaction was started by transferring the vial to a water bath maintained at 37°C and stopped after 30 minutes by injecting 0.5 ml of trichloroacetic acid (50%, w/v). To ensure complete trapping of H_2S, gas flow was continued for an additional 30 minutes.

The H_2S level was detected by a colorimetric method as described previously (Gil et al., 2011; Linden et al., 2008). The content in the second vial was moved to a test tube. Distilled water (3.5 ml), 0.4 ml of N,N-dimethyl-p-phenylene-diamine sulfate (20 mM) in HCl (7.2 M) and 0.4 ml of $FeCl_3$ (30 mM) in HCl (1.2 M) were added to the tube. The absorbance of the resulting solution at 670 nm was measured with a spectrophotometer (Hach, Loveland, CO, USA) after incubation at room temperature for 20 minutes. The calibration curve of absorbance versus the sulfide concentration was created by using the defined concentrations of NaHS solution, and the concentrations of H_2S were calculated and shown in $nmol \cdot min^{-1} \cdot g^{-1}$ tissue.

Immunofluorescence Staining. The colon was fixed with 10% formaldehyde for 24 hours, embedded in paraffin, and cut into 4-μm-thick sections. Then, the sections were blocked with 10% goat serum for one hour at room temperature. Specimens were incubated with rabbit anti-CBS (1:250) and anti-CSE antibodies (1:500) for 24 hours at 4°C, followed by three washes of five minutes in PBS. The sections were then incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (1:250) for 1 hour in the dark at room temperature. 4,6-Diamidino-2-phenylindole was used to stain nuclei. Sections in which primary antibodies were omitted in the same procedure were considered controls. After washing with PBS for 3 × 5 minutes, sections were visualized using a fluorescence microscope (Eclipse Ti-E, Nikon, Japan).

Western Blot Assay. Full-thickness colon tissues were extracted using radioimmunoprecipitation assay lysis buffer containing a protease and phosphatase inhibitor cocktail on ice with a homogenizer. The protein concentration in the supernatants was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded onto 12% gels, separated by SDS–PAGE, and then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA) at 280 mA for 1.5 hours. The membranes were blocked for 2 hours in 5% nonfat milk in Tris-buffered saline/Tween 20 at room temperature to block nonspecific binding and then incubated with rabbit polyclonal anti-CBS (1:500) and rabbit anti-CSE (1:500) primary antibodies overnight at 4°C. After washing five times in Tris-buffered saline/Tween 20, the membranes were incubated with a horseradish peroxidase-bound anti-rabbit secondary antibody (1:2000) for 1 hour at room temperature and washed with Tris-buffered saline/Tween 20for 3 × 5 minutes. Finally, protein bands were visualized using an enhanced chemiluminescence substrate. The

data are expressed as the band intensity ratios of the target proteins to glyceraldehyde-3-phosphate dehydrogenase.

Cell Isolation and Patch-Clamp Recording. The colon was dissected and pinned out in a Petri dish with ice-cold, oxygenated Ca^{2+}-free, HEPES-buffered saline (PBS) containing 135 mM of NaCl, 5.0 mM of KCl, 15.5 mM of NaOH, 1.2 mM of MgCl2, 10 mM of glucose, and 10 mM of HEPES (pH 7.4 adjusted with NaOH). The mucosa and submucosa were carefully removed with ophthalmic forceps, and then, the muscularis was cut into small strips and equilibrated in ice-cold Ca^{2+}-free PBS for 30 minutes. The strips were then incubated for 15–20 minutes at 36.8°C in an oxygenated enzyme solution containing 1.8 mg·ml^{-1} of collagenase II, 1.6 mg·ml^{-1} of soybean trypsin inhibitor, and 2.5 mg·ml^{-1} of bovine serum albumin. After that, the tissues were washed three times with Ca^{2+}-free PBS and then agitated using a glass pipette until the solution became cloudy. Then, the isolated smooth muscle cells (SMCs) were transferred and stored at 4°C and used within 8 h.

The cell suspension was plated directly on the glass bottom of a recording chamber and visualized using an inverted microscope (Olympus, Japan), equilibrated for 10 minutes beforehand, and then washed with Tyrode's buffer to remove debris. Whole-cell voltage clamp recording was performed using an Axopatch 700B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) with a 16-bit A/D converter (Digidata 1322 A, Molecular Devices). Recording pipettes were made by borosilicate glass capillaries using a puller (Model p-97, Sutter Instrument, Novato, CA), and the pipette resistances were 4–7 MΩ. Current traces were filtered at 1 kHz. All data were digitized and acquired using pClamp software (Clampex 10.2, Molecular Devices). All experiments were performed at room temperature (23–25°C). Patch pipettes for BK channels were filled with solution containing 125 mmol/l of KCl, 4 mmol/l of $MgCl_2$, 10 mmol/l of HEPES, 10 mmol/l of EGTA, and 5 mmol/l of Na_2 ATP (pH 7.3). The pipette solution for K_V channels contained 110 mM of potassium-aspartic acid, 5 mM of Mg-ATP, 5 mM of HEPES, 1 mM of $MgCl_2$, 20 mM of KCl, 10 mM of EGTA, 1 mM of $CdCl_2$, 2.5 mM of di-tris-creatine phosphate, and 2.5 mM of di-sodium-creatine phosphate, adjusted to pH 7.3 with KOH. Patch pipettes for L-type calcium channels were filled with solution containing 135 mmol/l of CsCl, 4 mmol/l of $MgCl_2$, 10 mmol/l of HEPES, 2 mmol/l of Na_2ATP, 10 mmol/l of EGTA, and 20 mmol/l of TEA (pH 7.3).

Statistical Analysis. The data were analyzed with GraphPad Prism software (version 8.4.3). All data are expressed as the mean ± S.D. In the present study, N values refer to the number of mice, and n values refer to the number of individual SMCs. The statistical significance between two groups was assessed by two-tailed Student's *t tests*. Multiple comparison analysis was performed by one-way ANOVA followed by Dunnett's test. Findings with $P < 0.05$ were accepted as statistically significant.

RESULTS

Blood Glucose, Body Weight and Fecal Pellet Expulsion in STZ-Induced Diabetic Mice. After four weeks of STZ injection, blood glucose levels were significantly elevated in diabetic mice compared with their controls (23.59 ± 3.59 mmol/L versus 5.04±0.580 mmol/L, $P < 0.0001$, $N = 10$) (Fig. 1A). The STZ-treated mice also showed lower body weight (STZ 31.5 ± 3.5 g versus control 39.3 ± 3.6 g, $P < 0.0001$) and decreased fecal pellets (STZ 1.6 ± 0.7 versus control 3.9 ± 1.0 g, $P < 0.0001$) (Fig. 1, B and C).

Spontaneous Contractility of Colonic Muscle Strips in Diabetic Mice. The LM and CM strips taken from both control and diabetic mice exhibited spontaneous activity after equilibration. In the control group, the contraction amplitude of LM strips was 0.81 ± 0.26 g, which was significantly greater than that in diabetic mice ($p = 0.0176$, $N = 10$). The contraction frequency of LM strips from normal mice was 0.56 ± 0.20 cpm, which was not significantly different from the diabetic group ($P > 0.05$) (Fig. 2, A and C).

A similar trend was observed in CM strips. As shown in Fig. 2, B and D, the mean amplitudes of the CM strips in the STZ group and control group were 0.51±0.16 g and 0.35±0.13 g, respectively ($p = 0.0250$, $N = 10$). No significant difference was observed in the contraction frequency of CM strips between the two groups (1.04±0.41 cpm versus 0.98 ± 0.34 cpm, $P > 0.05$) (Fig. 2, B and D).

Expression of CBS and CSE in the Colon of STZ-Induced Diabetic Mice. As shown in Fig. 3, A and B, both CSE and CBS were highly expressed in the mucosa and submucosa, whereas only CSE appeared to be intensely expressed in the muscle layers, including the myenteric plexus, of the colon from both diabetic mice and healthy controls. As shown in Fig. 4, A and B, STZ treatment resulted in upregulated expression of the two H_2S-generating enzymes in the colonic tissues ($P = 0.0191$ for CSE, $P = 0.0374$ for CBS versus control).

H_2S Production was Increased in the Colon of STZ-Induced Diabetic Mice. As shown in Fig. 5, the level of H_2S generated by colon in STZ-treated mice was markedly increased compared with that in the normal mice (10.53 ± 1.92 nmol·min^{-1}·g^{-1} versus 13.22 ± 2.11 nmol·min^{-1}·g^{-1}, $P = 0.0282$). The level of H_2S was 10.12 ± 1.74 nmol·min^{-1}·g^{-1} after incubation with L-propargylglycine and AOAA ($P > 0.05$ versus control, $P = 0.0111$ versus STZ).

Exogenous H_2S Exerted a Dual Effect on Colonic Contraction in Normal Mice. The effect of NaHS, an H_2S donor, on spontaneous contraction of colonic muscle strips was studied. As shown in Fig. 6, A and C, there was no significant change in either the amplitude or contraction frequency of LM strips after NaHS (0.02 mM) application ($P > 0.05$ versus control, $N = 7$). The amplitude of spontaneous contraction of LM strips after 0.1 mM of NaHS was significantly increased from 0.78 ± 0.22 g to 1.02 ± 0.19 g ($P = 0.0040$ versus control), and the contraction frequency increased from 0.49 ± 0.07 cpm to 0.89 ± 0.16 cpm ($P = 0.0036$ versus control). However, 0.5 mM and 1.5 mM of NaHS greatly suppressed the contraction of LM strips. The amplitude was reduced to 0.57 ± 0.17 g ($P = 0.0172$ versus control) and 0.26 ± 0.15 g ($P = 0.0115$ versus control), respectively. NaHS (0.5 mM) increased the contraction frequency to 0.98 ± 0.36 cpm ($P = 0.0448$ versus control), while 1.5 mM NaHS reduced the frequency to 0.09 ± 0.16 cpm ($P = 0.0020$ versus control).

In CM strips, NaHS (0.02 mM) had no effect on either amplitude or contraction frequency ($P > 0.05$ versus control, $N = 7$). NaHS (0.1 mM) increased the amplitude from 0.47 ± 0.19 g to 0.67 ± 0.12 g ($P = 0.0037$ versus control) but had no effect on the contraction frequency ($P > 0.05$ versus control). NaHS (0.5 mM and 1.5 mM) attenuated spontaneous contractile activities and reduced the amplitude to 0.36 ± 0.10 g ($P > 0.05$ versus control) and 0.13 ± 0.12 g ($P = 0038$ versus control), respectively. A similar trend was observed after TTX (1 μM) pretreatment (Fig. 6, B and D).

Biphasic Effect of Exogenous H2S on Membrane Currents of VDCCs. Fig. 7A shows the raw traces of currents from VDCCs elicited by step depolarization with 300-millisecond pulses from a holding potential of -50 mV to +40 mV in 10 mV steps before and after NaHS administration (0.1, 0.5, and 1.5 mM). Fig. 7B shows the averaged current-voltage relationships (I-V) of the VDCCs in the presence and absence of NaHS. NaHS (0.1 mM) had no influence on the shape of the I-V curve,

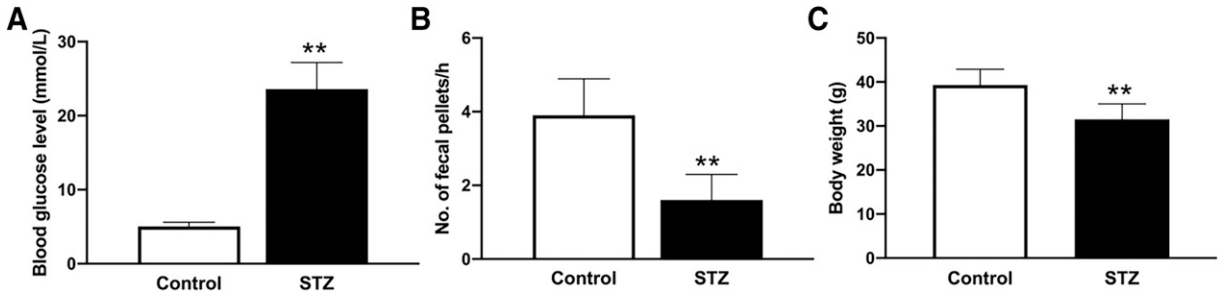

Fig. 1. Blood glucose, body weight, and fecal pellet expulsion in STZ-induced diabetic mice: blood glucose levels (A), body weight (B) and number of fecal pellets (C) in induced diabetic mice and normal controls at four weeks after STZ treatment. (**$p < 0.0001$ versus Control, according to an unpaired Student's t test, $N = 10$).

while NaHS at 0.5 and 1.5 mM caused a significant right shift. The voltage at the maximal value of the peak membrane currents increased from 0 mV to 10 mV and 20 mV, respectively. NaHS (0.1 mM) increased the current density at 0 mV from −3.69 ± 0.88 pA/pF to −4.44 ± 1.00 pA/pF ($P = 0.0245$ versus control), which was reversed after application of 0.5 mM and 1.5 mM of NaHS. The current density decreased to -3.94 ± 0.49 pA/pF ($P > 0.05$ versus control) and -1.64 ± 1.20 pA/pF ($P = 0.0106$ versus control), respectively (Fig. 7C).

3.7. Inhibitory Effect of Exogenous H_2S on Currents of K_V Channels. Fig. 8A shows the original traces of currents from K_V channels elicited by step depolarization with 500-millisecond pulses from a holding potential of -80 mV to +60 mV in 20-mV steps before and after NaHS application (0.1, 0.5, and 1.5 mM). Fig. 8B shows the averaged current-voltage (I-V) relationships of K_V channels in the presence and absence of NaHS. NaHS concentration dependently decreased the membrane currents of K_V channels. The current density at 60 mV was reduced from 16.37 ± 4.11 pA/pF to 15.95 ± 3.66 pA/pF ($P > 0.05$ versus control), 14.95 ± 3.58 pA/pF ($P = 0.0026$ versus control) and 13.29 ± 2.31 pA/pF ($P = 0.0223$ versus control) (Fig. 8C).

Effect of Exogenous H_2S on BK Channel Currents. Fig. 9A shows the raw traces of currents from BK channels elicited by step depolarization with 400-millisecond pulses from a holding potential of -80 mV to +60 mV in 20-mV steps before and after NaHS administration (0.1, 0.5, and 1.5 mM). Fig. 9B shows the averaged I-V relationship of BK channels in the presence and absence of NaHS. Application of NaHS caused a remarkable reduction in the membrane currents in a dose-dependent manner. The current density at 60 mV was reduced from 18.00 ± 2.67 pA/pF to 16.96 ± 3.00 pA/pF ($P = 0.0096$ versus control), 15.53 ± 3.12 pA/pF ($P = 0.0002$ versus control) and 14.27 ± 3.28 pA/pF ($P = 0.0002$ versus control) (Fig. 9C).

Discussion and Conclusion

Colonic motility disturbance is a well-documented complication in various forms of diabetes (Feldman and Schiller, 1983; Maleki et al., 1998; Nakahara et al., 2002). However, the pathophysiology behind diabetes-related colonic dysmotility remains incompletely understood. H_2S, a novel gaseotransmitter, has been found to regulate gut motility in a biphasic dose–response manner [12,13]. While several studies have shown that changes

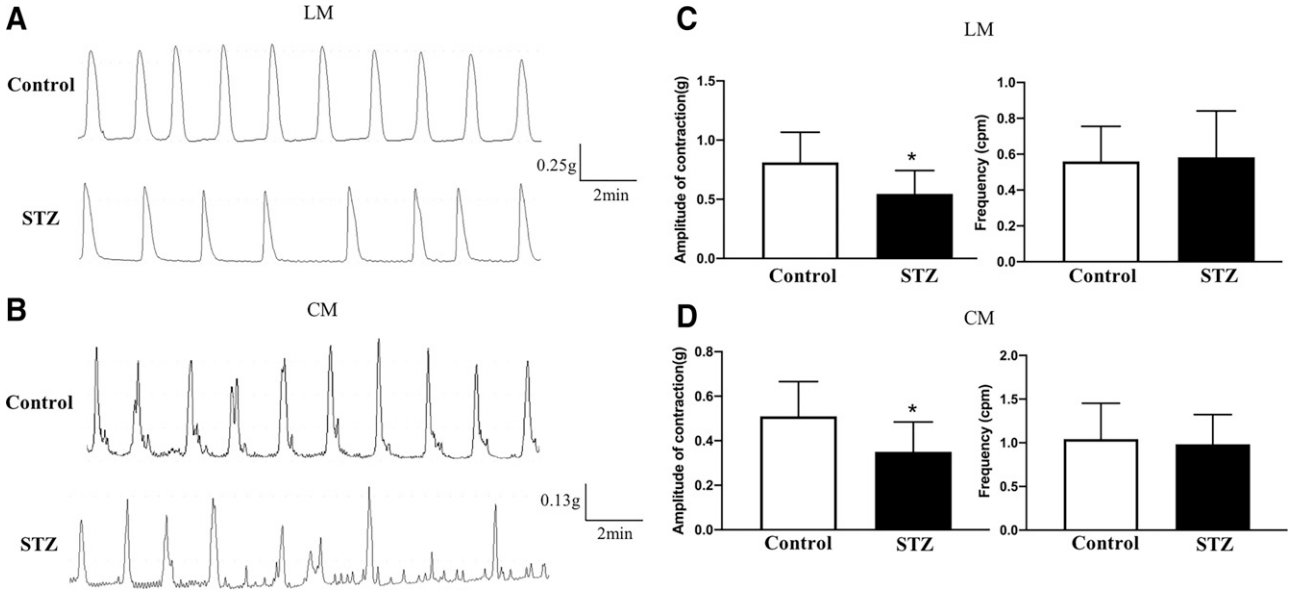

Fig. 2. Spontaneous contractile activities of colonic muscle strips in diabetic mice: (A) and (B Trace showing a comparison of spontaneous contractile activities of the colonic muscle strips in control and diabetic mice. (C) and (D) Summarized data of the amplitude and contraction frequency of LM and CM strips in STZ-treated and control mice. (*$p < 0.05$, **$p < 0.001$ versus Control, according to an unpaired Student's t test, $N = 10$).

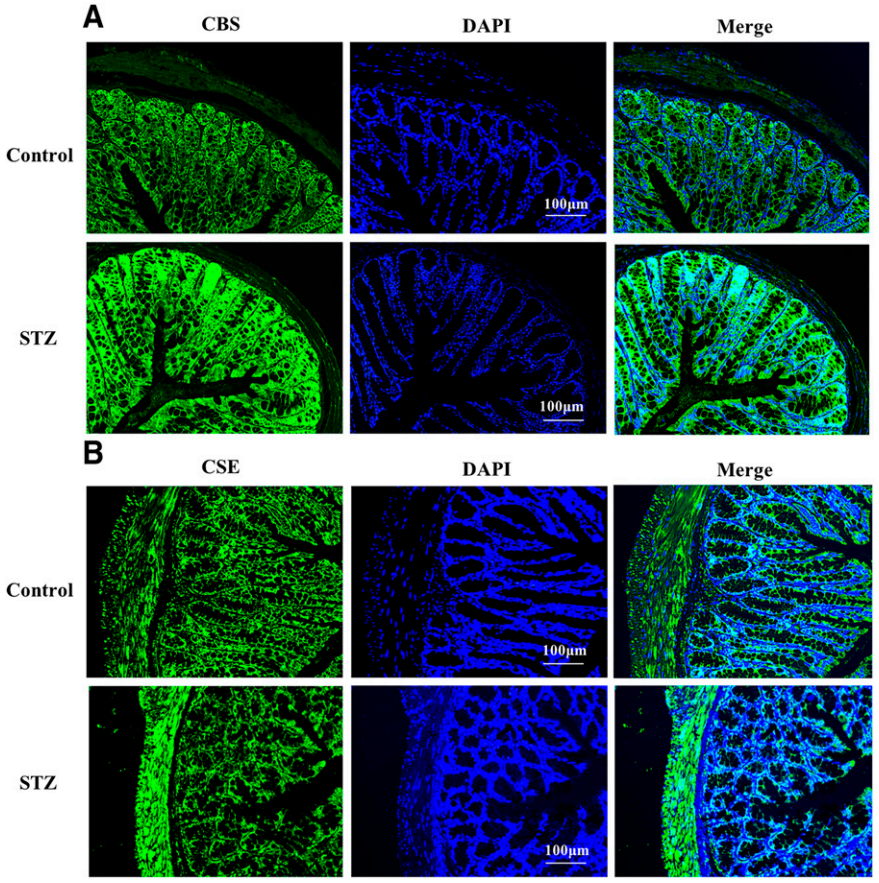

Fig. 3. Distribution of CBS (A) and CSE (B) in the colon of diabetic mice and control mice. (original magnification ×200, scale bar 100 μm).

in H_2S homeostasis play an important role in the pathogenesis of diabetes (Szabo, 2012; Yusuf et al., 2005), there are no published data thus far on the potential involvement of H_2S in colonic dysmotility linked to diabetes. In the current study, we found that diabetic mice exhibited greatly decreased spontaneous contractions of colonic muscle strips compared with the control mice. The expression levels of H_2S and H_2S synthetase in the colon from diabetic mice were markedly increased. In addition, exogenous H_2S exerted biphasic effects, including excitation at lower concentrations and inhibition at higher levels, on the spontaneous contraction of colonic smooth muscle. To our knowledge, this is the first study to demonstrate that H_2S overproduction with subsequent suppression of muscle contraction may contribute in part to the pathogenesis of colonic hypomotility in DM.

Increasing evidence shows that STZ-induced diabetic mice exhibit decreased colonic motility (Touw et al., 2012; Song et al., 2018). The results from our present study further illustrate that STZ-induced diabetic mice showed decreased stool output and impaired colonic contractility, which was consistent with human studies (Klinge et al., 2020). Conversely, diabetic rats induced by STZ have been reported to show enhanced spontaneous contractile activity in the colon (Forrest and Parsons, 2003). Different species and different severities of DM may contribute to this discrepancy.

In the colon, both CSE and CBS are involved in the generation of H_2S (Jimenez et al., 2017; Gil et al., 2011). In the present study, we identified that both CSE and CBS were highly expressed in the mucosa, whereas only CSE appeared to be

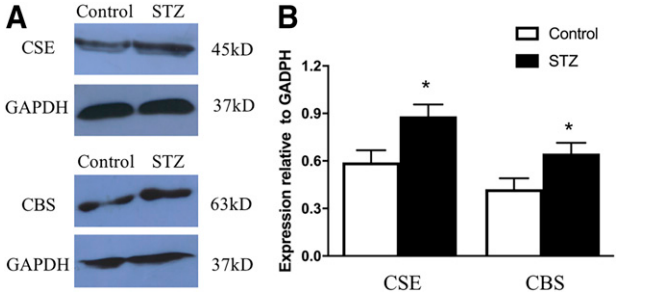

Fig. 4. Expression of CBS and CSE in the colon of diabetic mice: (A) Representative traces of the expression of CSE and CBS in diabetic mice and normal mice. (B) Summarized results showing that the expression of H2S-producing enzymes was significantly decreased in STZ-treated mice. (*$p < 0.05$ versus control, according to an unpaired Student's t test, $N = 7$).

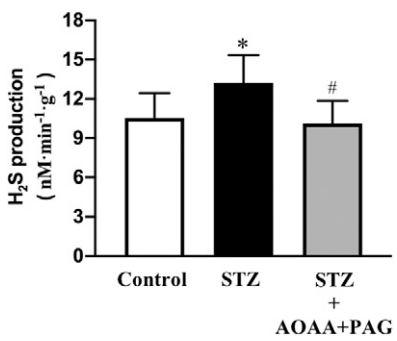

Fig. 5. H_2S production was increased in the colon of STZ-induced diabetic mice. (*$p < 0.05$ versus Control, $^{\#}p < 0.05$ versus STZ, according to a one-way ANOVA followed by Dunnett's test $N = 8$).

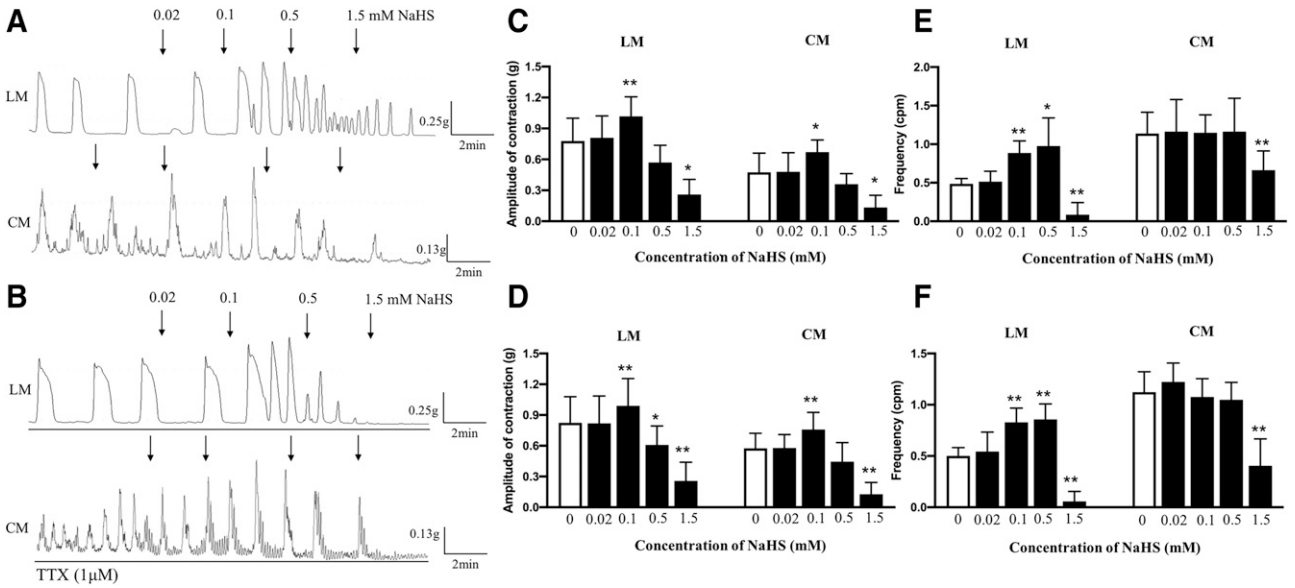

Fig. 6. Dual effects of exogenous H_2S on spontaneous contraction of colonic smooth muscle from normal mice: (A) and (B) Original traces of spontaneous contractions of the longitudinal LM and CM of colon from normal mice after NaHS application at different concentrations in the presence and absence of TTX. (C) and (D) Summary of the effects of NaHS on contractile activities of both LM and CM strips. € and (F) Summary of the effects of NaHS on contraction frequency of both LM and CM strips. (*$p < 0.05$, **$p < 0.01$ versus baseline, according to a paired Student's t test, $N = 7$).

intensely expressed in the muscle layers, including the myenteric plexus. Similar staining was described in a previous study (Linden et al., 2008). However, the very weak expression of CBS in the muscle layers and myenteric neurons was not consistent with previous studies from rat, guinea pig and human colon (Gil et al., 2011; Schicho et al., 2006). Differences in the distribution of the enzymes may be due to different technical approaches and species differences. The present study showed that CSE and CBS expression in intact colons were both upregulated in diabetic mice. Furthermore, H_2S production was significantly increased in STZ-treated mice, which was effectively blocked by a combination of L-propargylglycine and AOAA. Decreased intestinal barrier function has been reported in patients with DM and in experimental models of DM (Bosi et al., 2006; Meddings et al., 1999). Thus, more H_2S produced from CSE and CBS in the mucosa could access the muscle layer and inhibit muscle contraction in diabetic mice. However, there are conflicting reports in the literature as to whether CBS and CSE are upregulated in diabetes. It has been reported that no notable changes were observed in the expression of CBS or CSE in the brain, heart, kidneys, and lungs of diabetic rats (Suzuki et al., 2011). It is possible that these differences are tissue and species dependent. Taken together, the data presented in this study further illustrate that both CBS

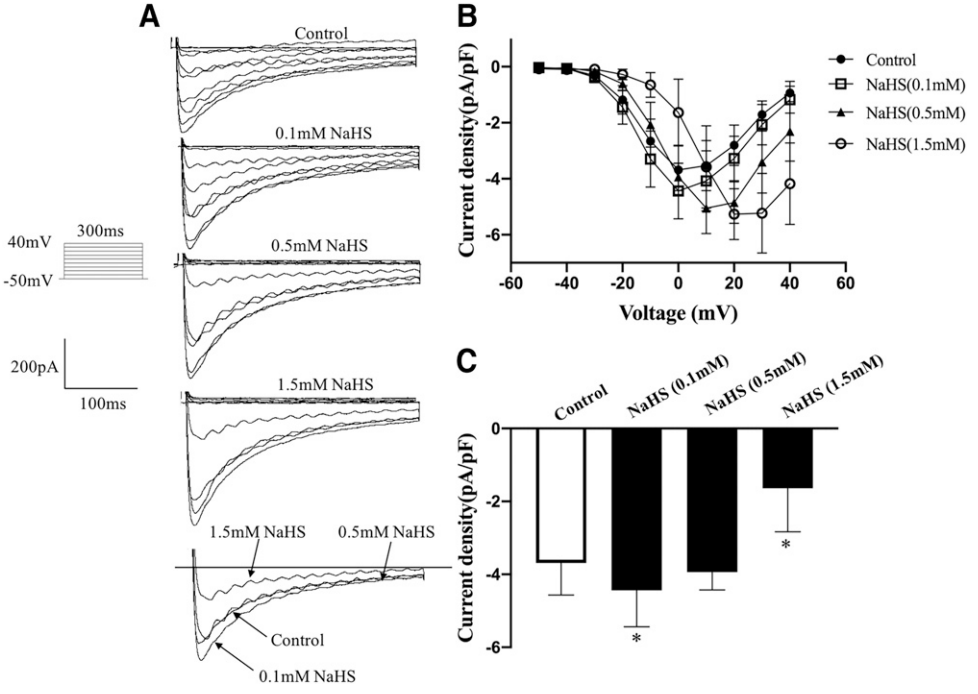

Fig. 7. Biaphisic effect of exogenous H_2S on the currents of VDCC channels: Original traces of VDCCs elicited by a series of 300-millisecond depolarizing voltage pulses from a holding potential of −50 mV to +40 mV in 10-mV steps before and after NaHS administration. (B) Change in the current-voltage relationship of VDCCs before and after NaHS application. (C) Summarized data showing the effects of NaHS on the peak current density of VDCCs at 0 mV. (*$p < 0.05$ versus control, according to a paired Student's t test, $N = 7$, $n = 8$).

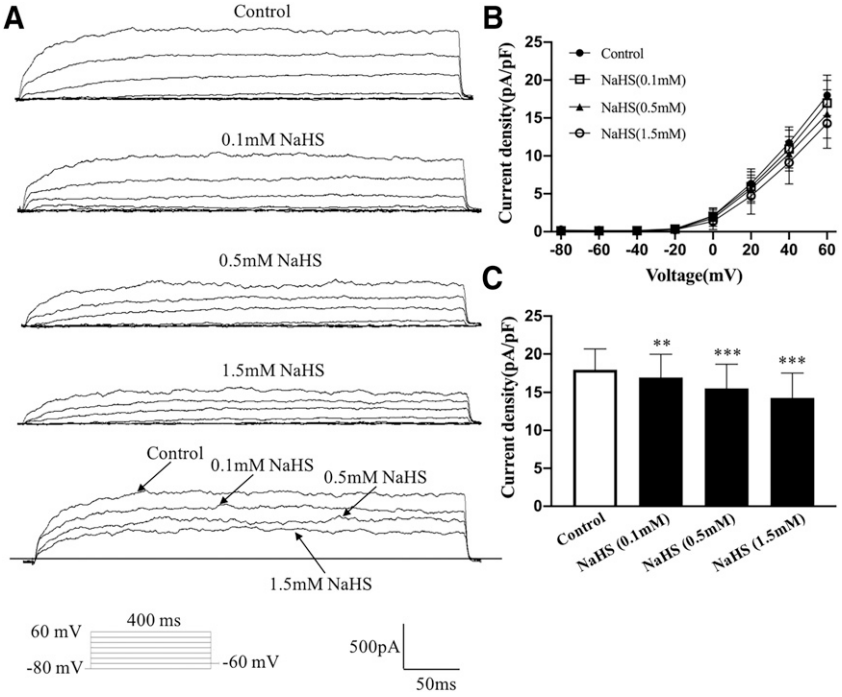

Fig. 8. Inhibitory effect of exogenous H_2S on membrane currents of K_V channels: Original traces of K_V channels elicited by a series of 500-millisecond depolarizing voltage pulses from a holding potential of −80 mV to +60 mV in 20-mV steps before and after NaHS administration. (B) Change in the current-voltage relationship of K_V channels before and after NaHS application. (C) Summarized data showing the effects of NaHS on the current density of Kv channels at 60 mV. ($*p < 0.05$, $**p < 0.01$ versus control, according to a paired Student's t test, $N = 8$, $n = 8$).

and CSE participate in the endogenous generation of H_2S in the mouse colon and that elevated production of H_2S may play a role in the colonic hypomotility of diabetic mice.

In the present study, the effects of exogenous H_2S on spontaneous contraction of colonic muscle strips were observed. In line with previous studies (Zhao et al., 2009; Han et al., 2011), we found that NaHS elicited a biphasic action on muscle contraction, including excitation at lower concentrations (0.02–0.1 mM), as well as inhibition at higher levels (>0.5 mM). The actual yield of H_2S is 33% of the amount of NaHS (Reiffenstein et al., 1992). NaHS (0.02–0.1 mM) may produce approximately 6–33 μM H_2S, while NaHS (0.5-1.5 mM) possibly releases approximately 160–450 μM H_2S. It has been reported that the physiologic concentration of H_2S in mammalian tissue and blood is 1–160 μM (Bhatia, 2005). Thus, the dual effects of H_2S observed in the present study may be a physiologic action. However, in contrast to our study, several studies have characterized the inhibitory action of H_2S in the regulation of gut motility (Martinez-Cutillas et al., 2015; Kasparek et al., 2012). These conflicting results are presumably due to substantial differences in the experimental conditions employed and the biphasic nature of H_2S. Note that the inhibition induced by H_2S at higher levels (>160 μM) may be ascribed to cell cytotoxicity. Furthermore, the dual effects of NaHS on colonic muscle contraction were not blocked by TTX pretreatment, which indicates that the neural pathway may not be involved in the biphasic action of H_2S.

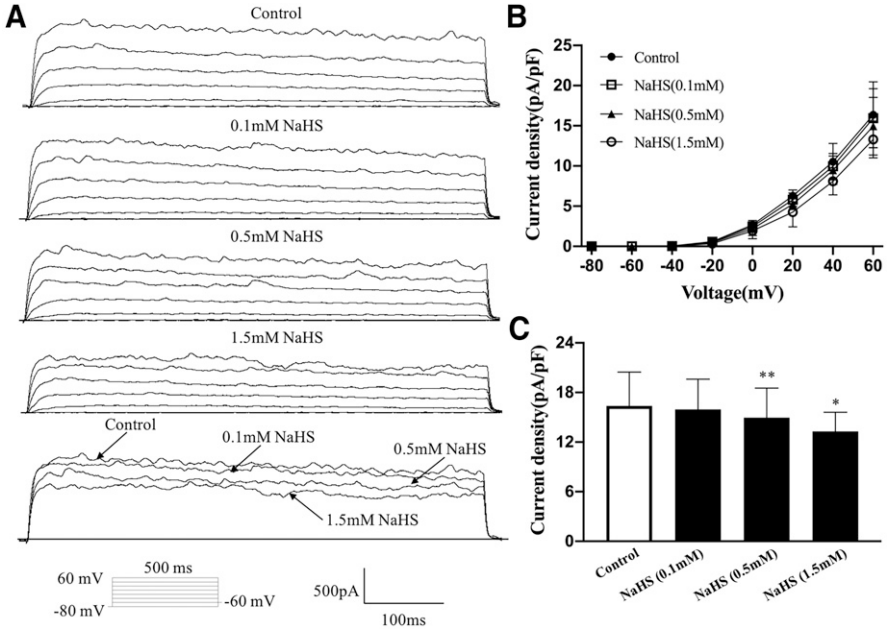

Fig. 9. Inhibitory effect of exogenous H_2S on BK channel currents: (A) Original traces of BK channels elicited by a series of 400-millisecond depolarizing voltage pulses from a holding potential of −80 mV to +60 mV in 20-mV steps before and after NaHS administration. (B) Change in the current-voltage relationship of BK channels before and after NaHS application. (C) Summarized data showing the effects of NaHS on the current density of BK channels at 60 mV. ($**p < 0.01$, $***p < 0.001$ versus Control, according to a paired Student's t test, $N = 8$, $n = 10$).

Increasing evidence suggests that many biologic effects of H_2S are, either in part or in whole, mediated by ion channels (Zhao et al., 2009; Han et al., 2011; Zhao et al., 2001; Meng et al., 2015; Telezhkin et al., 2009). In addition to ATP-sensitive potassium (K_{ATP}) channels, which are the first reported molecular targets for H_2S, H_2S is also known to act on a number of other ion channels. It has been reported that H_2S inhibits K_V channels in gastric smooth muscle and inhibits BK channels in stably transfected HEK-293 cells [12,38,39]. Furthermore, H_2S activates VDCCs in gastric SMCs [39]. These ion channels, individually or collectively, also participate in regulating the contraction of SMCs in the GI tract (Bolton et al., 1999). In the present study, we found that NaHS exhibited a dual effect on VDCCs, with activation at lower concentrations (0.1 mM) and inhibition at higher concentrations (0.5–1.5 mM), which may contribute to the biphasic action of NaHS on the contraction of muscle strips recorded in the organ bath. NaHS at high concentrations moved the I-V curve of VDCCs to the right, which indicates that high-dose NaHS may modify the voltage-dependent properties of VDCCs. Furthermore, NaHS inhibited the currents of both K_V channels and BK channels in a concentration-dependent manner, consistent with previous studies (Zhao et al., 2009; Meng et al., 2015; Telezhkin et al., 2009). Suppression of the two potassium channels results in cell membrane depolarization with subsequent increased Ca^{2+} entry and smooth muscle cell contraction, which may contribute in part to the excitatory response to NaHS. The question then arose: why was excitation of muscle contraction induced by NaHS at higher concentrations not observed in the organ bath experiment? It is known that the strength of contractions generated by smooth muscle is mainly determined by the amplitude, duration, and frequency of action potentials, which are mediated primarily through VDCCs (Wegener et al., 2006). Thus, we suspected that suppression of VDCCs plays a dominant role in NaHS-elicited actions on different ion channels at high concentrations, followed by relaxation of muscle contraction. However, conflicting results regarding the effects of NaHS on these ion channels have been reported in previous studies (Tang et al., 2010). The diverse characteristics of different cell types and the biphasic nature of the pharmacological actions of H_2S may contribute to this discrepancy.

It is well known that H_2S exerts many of its biologic effects by targeting proteins for S-sulfhydration, which is analogous to the S-nitrosylation of nitric oxide (Tang et al., 2010). Various ion channels contain reactive cysteine residues that can be modified through S-sulfhydration, resulting in the formation of hydropersulfide, including examples from K_{ATP} channels and VDCCs (Zhao et al., 2001; Zhang et al., 2012). Therefore, the actions of NaHS presented in this study may contribute to S-sulfhydration with subsequent alterations in the three-dimensional structure, functional state, and quantity of these ion channels in an activated or inactivated state.

In conclusion, spontaneous contractility of colonic smooth muscle from diabetic mice induced by STZ was remarkably attenuated, which may underlie the constipation associated with DM. H_2S overproduction with subsequent suppression of muscle contraction via VDCCs on SMCs may contribute in part to the pathogenesis of colonic hypomotility in DM. Inhibition of colonic H_2S biosynthesis could be a potential approach to alleviate constipation in patients with DM.

Authorship Contributions

Participated in research design: Luo, Wang.
Conducted experiments: Wang.
Contributed new reagents or analytic tools: Zhang.
Performed data analysis: Quan, Liu, Sun.
Wrote or contributed to the writing of the manuscript: Quan.

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