Hydrogen sulphide suppresses human atrial fibroblast proliferation and transformation to myofibroblasts

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Abstract

Cardiac fibroblasts are crucial in pathophysiology of the myocardium whereby their aberrant proliferation has significant impact on cardiac function. Hydrogen sulphide (H2S) is a gaseous modulator of potassium channels on cardiomyocytes and has been reported to attenuate cardiac fibrosis. Yet, the mechanism of H2S in modulating proliferation of cardiac fibroblasts remains poorly understood. We hypothesized that H2S inhibits proliferative response of atrial fibroblasts through modulation of potassium channels. Biophysical property of potassium channels in human atrial fibroblasts was examined by whole-cell patch clamp technique and their cellular proliferation in response to H2S was assessed by BrdU assay. Large conductance Ca2+-activated K+ current (BKCa), transient outward K+ current (Ito) and inwardly rectifying K+ current (IKir) were found in human atrial fibroblasts. Current density of BKCa (IC50 = 69.4 µM; n = 6), Ito (IC50 = 55.1 µM; n = 6) and IKir (IC50 = 78.9 µM; n = 6) was significantly decreased (P < 0.05) by acute exposure to NaHS (a H2S donor) in atrial fibroblasts. Furthermore, NaHS (100–500 µM) inhibited fibroblast proliferation induced by transforming growth factor-β1 (TGF-β1; 1 ng/ml), Ang II (100 nM) or 20% FBS. Pre-conditioning of fibroblasts with NaHS decreased basal expression of Kv4.3 (encode Ito), but not KCa1.1 (encode BKCa) and Kir2.1 (encode IKir). Furthermore, H2S significantly attenuated TGF-β1-stimulated Kv4.3 and α-smooth muscle actin expression, which coincided with its inhibition of TGF-β-induced myofibroblast transformation. Our results show that H2S attenuates atrial fibroblast proliferation via suppression of K+ channel activity and moderates their differentiation towards myofibroblasts.

Keywords: fibroblast • potassium channel • hydrogen sulphide • atrial fibrosis

Introduction

Cardiac fibroblasts are fundamentally involved in cardiac remodelling in normal ageing heart [1] and in damaged myocardium [2]. Ablent proliferation of fibroblasts and their transformation to myofibroblasts is a hallmark of cardiac fibrosis, which is characterized by excessive extracellular matrix built-up leading to loss of tissue compliance [3, 4]. Because of their wide-ranging participation in myocardial pathophysiology, cardiac fibroblasts represent an attractive target in managing cardiac disorders, including cardiac hypertrophy, heart failure and arrhythmias [5]. Indeed, atrial fibrosis has been closely associated with atrial fibrillation [6, 7] and sinus node dysfunction [8].

Hydrogen sulphide (H2S) is an endogenously generated gaseous transmitter that has been reported to attenuate cardiac fibrosis [9]. It is known to mediate its effects by modulating ion channel activity in many cellular systems [10]. Hydrogen sulphide was the first opener of KATP channel identified in vascular smooth muscle cells [11]. Through activation of KATP channels, H2S lowers blood pressure, protects heart from ischaemia and reperfusion injury [12, 13]. We have recently reported that H2S inhibited delayed rectifier potassium channels in human iPSC-derived cardiomyocytes [14]. Yet, effect of H2S on cardiac fibroblasts remains poorly understood. We hypothesized that...
H2S inhibits proliferation of atrial fibroblasts by inhibiting functioning of potassium channels. We present supporting data that H2S may potentially modulate cardiac fibrosis by inhibiting BKCa, Iks, and IKir, independent of KATP channels, leading to decreased proliferation and suppression of transforming growth factor-β1 (TGF-β1)-induced myofibroblast transformation of atrial fibroblasts.

Materials and methods

Fibroblast isolation

Patients undergoing mitral valve repair and coronary bypass surgery (n = 10) were recruited after informed consent in protocol approved by institutional review board of Singapore General Hospital that conformed to the Declaration of Helsinki. Atrial appendages were collected as surgical by-product. Human atrial fibroblasts were isolated by mincing the appendages to less than 1 mm³ and followed by 0.1% trypsin digestion for 20 min. before plating onto tissue culture–treated 60-mm dishes to produce fibroblastic outgrowth from minced tissue pieces. The isolated fibroblasts were confirmed with expression of collagen I (1/20; Southern Biotech, Birmingham, AL, USA) and anti-human fibroblast (1/1000; Sigma-Aldrich, St. Louis, MO, USA) antibodies (Fig. S1). Atrial fibroblasts were passaged as monolayer in 10% foetal bovine serum (1/1000; Sigma-Aldrich, St. Louis, MO, USA) antibodies (Fig. S1).

Electrophysiological recordings

Cell were placed on the stage of a Nikon Diaphot inverted microscope and superfused continuously at 36 ± 1°C with Tyrode solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 10 HEPES and 10 Glucose (pH adjusted to 7.4 with NaOH). The patch-clamped cell was superfused by means of a temperature-controlled micro-supercilor (TC-324B, Warner Instruments, Hamden, CT, USA). Patch pipettes were made from borosilicate glass shanks (Sutter Instrument, Novato, CA, USA) and pulled with a Brown–Flaming puller (Model P-97; Sutter Instrument Co), and had tip resistances of 2–3 MΩ when filled with pipette solution. Pipette tips were polished (Microforge MF630; Narishige, Tokyo, Japan). These patch pipettes were filled with a standard solution containing (in mM) 140 KCl, 1.2 MgCl2, 0.05 EGTA, 10 HEPES, 0.1 GTP and 5.0 Mg ATP (pH adjusted to 7.2 with KOH). For Na+ current recording, the patch pipettes were filled with (in mM) 35 NaCl, 105 CsF, 0.1 EGTA and 10 HEPES (pH adjusted to 7.4 with CsOH). After a gigaohm seal was obtained by negative pressure suction, the cell membrane was ruptured by a gentle suction to establish whole-cell configuration with a seal resistance >800 MΩ. The cell membrane capacitance (40.27 ± 8.2 pF) was electrically compensated with the pulse software. The series resistance (Rs, 3–5 MΩ) was compensated by 50–70% to minimize voltage errors. Currents were elicited with voltage protocols as described in the following results section for different individual current recordings. Whole-cell voltage-clamp experiments were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) interfaced to a Digidata 1322A data acquisition system controlled by Clampex version 8.1 software (Axon Instruments). Data were analysed with pCLAMP software (Version 10.0; Axon Instrument) and Origin 8.0 (OriginLab, Northampton, MA, USA).

Cell proliferation and apoptosis assay

Cell proliferation assay was performed with BrdU kit (Roche, Basel, Switzerland). Briefly, cells were plated on 96-well plate at a density of 3000/well and cultured for 24 hrs. After 4 hrs of serum starvation, cells were incubated for 24 hrs with medium containing ion channel blockers, NaHS or growth factors. BrdU labelling solution (100 μM) diluted 10 times in DMEM (0.1% FBS) was added to each well and the plates were incubated at 37°C for an additional 2 hrs. Incorporated BrdU was detected by an anti-BrdU antibody for 90 min. and colorimetric development proceeded for 15 min. before analysis by ELISA plate reader (SpectraMax, Molecular Device, Sunnyvale, CA, USA). Cellular apoptosis assay was performed with Caspase-3 Fluorescence Assay kit as instructed (Cayman Chemical, Ann Arbor, MI, USA). Briefly, cells were plated on 96-well plate at a density of 10³/well and cultured for 24 hrs. After 4 hrs of serum starvation, cells were incubated for 24 hrs with medium containing NaHS. Fluorescent intensity was obtained with ELISA plate reader (SpectraMax, Molecular Device) at 485 nm excitation and 535 nm emission wavelengths.

RNA isolation and RT-PCR

Total RNA was extracted from human atrial fibroblasts with Trizol reagent (Life Technologies, Carlsbad, CA, USA) after 12 hrs of treatment. RT-PCR was performed with one-step kit (Invitrogen) where 1 μg RNA and random hexamer primer were used for the initiation of cDNA synthesis. Gene-specific primers for the BKCa (Kcα1.1): forward 5′-GGAG-GATGCTCTGAAATATCA-3′; reverse 5′-AGCTCGGATGTTTAGCAAGA-3′; Iks (Kv4.3): forward 5′-CTGGACAA GAA CACGCCAGTGG-3′; reverse 5′-ATCACG ATCAGGAAGGCA CATAAGG-3′ and IKir (Kv1.2): forward 5′-TTGAGACCCGACACAACTGATG-3′; reverse 5′-TGGGTTGACACTGCTGCAAATG-3′; x-SMA: forward 5′-CATACACCACTTGAGGACACA-3′; reverse 5′-GTGCGTGGACA CACATCCGCA-3′; CSE: forward 5′-TCCGGATGGA AAAGACTCT-3′; reverse 5′-GCTGGTTTAAAGGTGAC-3′; KATP (Kir6.2): forward 5′-GTGGTGAGACACCATCCTGCA-3′; reverse 5′-GATGGTTGGACCA AAGTC-3′; β-actin: forward 5′-TTTGTGACCTCCTTCCAACCC-3′; reverse 5′ TTGGTGAGGCA GCAAGA-3′; PCR products were fractionated on 2% agarose gel electrophoresis. Data were expressed as values of optical density (OD) standardized to those of β-actin.

Immunocytochemistry

Atrial fibroblasts cultured on LabTek chamber slides (Nunc; Thermo Fisher Scientific, Waltham, MA, USA) were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X100 and blocked with 2% BSA. Cells were incubated overnight with antibodies against α-smooth muscle actin (1/2000; α-SMA; Sigma-Aldrich) to identify myofibroblasts, against anti-Kv1.1 (1/1000; Abcam, Cambridge, UK), anti-Kv4.3 (1/500; Abcam) and anti-Kir2.1 (1/1000; Abcam) to identify BKCa, Iks and IKir channels, respectively (Fig. S2), before incubating with Alexa Fluor 488 or 555 secondary antibody (Life Technologies) and mounted in Vectashield mounting media containing DAPI for nuclear counterstain.
Statistical analysis

Data were expressed as mean ± SE. Statistical significance of the difference between groups was determined with Student’s t-test. A value of P < 0.05 was considered statistically significant.

Results

Hydrogen sulphide suppresses ion currents in human atrial fibroblasts

Multiple ionic channels are reported to be expressed in human cardiac ventricular fibroblasts [15], ionic channels in our atrial fibroblasts were activated by depolarization voltage between −70 and −60 mV from a holding potential of −80 mV (0.2 Hz) to elicit total outward K⁺ currents. Activated currents that were sensitive to paxilline (1 mM), a specific BKCa inhibitor, were significantly suppressed at +60 mV, confirming the presence of BKCa current (52%; 163/309 cells) in human atrial fibroblasts (Fig. 1A). Under identical voltage-clamp condition, exposure to 100 μM NaHS (as a donor of H₂S) similarly reduced the peak current density of BKCa (Fig. 1B). The inhibitory effects observed could not be washed out (Fig. 1C). The presence of NaHS resulted in a voltage-dependent suppression of the I–V curve from 10.5 ± 1.2 pA/pF to 6.8 ± 0.9 pA/pF at +40 mV (P < 0.01; n = 6) (Fig. 1D) and a dose-dependent inhibition of BKCa peak current density with an IC₅₀ of 69.4 μM (Fig. 1E).

To verify the specificity of H₂S inhibition on BKCa, we assessed its effect in the presence of naringenin (10 μM), a specific opener of BKCa [16]. BKCa currents were elicited with clamp pulses at +40 mV from a holding potential of −80 mV under control condition (Fig. 1F). Compared with baseline (9.9 ± 0.8 pA/pF), naringenin increased BKCa current significantly (14.1 ± 0.5 pA/pF; P < 0.01; n = 6), but addition of NaHS returned naringenin-induced current to baseline (9.2 ± 0.4 pA/pF; P < 0.05; n = 6) (Fig. 1G). The rising phase of the BKCa currents at 50 mV with activation τ (τₕ₉) at baseline (15.4 ± 0.1 ms) was lowered significantly by naringenin (8.6 ± 0.2 ms; P < 0.01; n = 6), but reversed to baseline after addition of NaHS (15.4 ± 0.2 ms; P < 0.05; n = 6), which confirmed its modulation of BKCa channel kinetics (Fig. 1H).

Similarly, under conditions to elicit total outward K⁺ currents, a 4-aminoypyridine (4-AP; 0.5 mM)–sensitive current was detected, indicating the presence of transient outward currents, Ito (34%; 104/309 cells) in the atrial fibroblasts (Fig. 2A). Under identical voltage-clamp condition, exposure of fibroblasts to 100 μM NaHS reduced the peak

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**Fig. 1** Effect of NaHS on BKCa currents in human atrial fibroblasts. (A) Voltage-dependent current was suppressed by BKCa blocker Paxilline (1 μM). Paxilline-sensitive I–V relationships of the membrane currents of typical BKCa channel. (B) BKCa traces recorded in the absence and presence of NaHS (100 μM). (C) Time course of BKCa current inhibition in human atrial fibroblast after addition of NaHS (100 μM). (D) Mean I–V relationship of peak BKCa current in the absence and presence of NaHS (100 μM) (**P < 0.01; *P < 0.05 versus control). (E) A concentration response curve of NaHS-induced inhibition on BKCa. (F) Effect of NaHS (100 μM) on BKCa currents in the presence of Naringenin (10 μM). (G) Summarized data for peak BKCa currents at +40 mV at baseline, in the presence of Naringenin (10 μM), and in the presence of NaHS (100 μM) (*P < 0.05 versus basal levels; †P < 0.05 versus Naringenin alone; n = 6). (H) Plot of the activation τ (τₚ₉) as a function of membrane potential in the presence of Naringenin (10 μM) and Naringenin together with NaHS (100 μM) (**P < 0.01 versus basal levels; †P < 0.05 versus Naringenin alone; n = 6).
The inhibitory effects occurred within 1 min., reached saturation by 10 min. and could not be washed out (Fig. 2C). Addition of NaHS showed a voltage-dependent suppression of the I\textsubscript{to} current in the I-V curve from 18.2 ± 1.5 pA/pF to 12.7 ± 1.7 pA/pF at +40 mV (P < 0.05; n = 6) (Fig. 2D) and demonstrated a dose-dependent inhibition of peak current density with an IC\textsubscript{50} of 55.1 nM (Fig. 2E).

Steady-state activation of I\textsubscript{to} was unaffected by NaHS (Fig. 2F). The curves were fitted by the Boltzman equation: $G/G_{max} = 1/[1 + \exp(V - V_{1/2}/\kappa)]$, where $G/G_{max}$ represents a ratio of conductance to the maximum conductance, and $V$ represents the values of the depolarizing pulses. The half-maximum activation voltage ($V_{1/2}$) and slope factor under control condition were 17.2 ± 1.5 mV and 19.3 ± 1.3, respectively, which were not significantly different from those in the presence of NaHS ($V_{1/2}$: 18.3 ± 1.2 mV, slope factor 20.2 ± 1.2) (F = NS; n = 6). In contrast, NaHS significantly influenced the steady-state inactivation of I\textsubscript{to} (Fig. 2G). When fitted to Boltzman function, $I/I_{max} = 1/[1 + \exp(V - V_{1/2}/\kappa)]$, the half-maximum inactivation voltage ($V_{1/2\text{inact}}$) and slope factor under control condition were −53.6 ± 1.2 mV and 9.08 ± 1.1, respectively, which were significantly different from those in the presence of NaHS ($V_{1/2\text{inact}}$: −71.1 ± 3.1 mV, slope factor 14.7 ± 2.4) (P < 0.05; n = 6). Furthermore, recovery of I\textsubscript{to} from inactivation was analysed by delivering two identical 500 ms depolarizing pulses from −80 to +60 mV and varying the interpulse from 50 to 3500 ms. Addition of NaHS shifted the curve right and increased the half-recovery time of I\textsubscript{to} from of 461.7 ± 57 to 1215.2 ± 49 ms. (P < 0.01; n = 6) (Fig. 2H), confirming inhibition...
of NaHS on the kinetic property of I\text{to} channel recovery. Furthermore, these properties of I\text{to} were similar to those reported in human ventricular fibroblasts [15].

The inhibitory effect of NaHS on I\text{to} was further confirmed in the presence of NS5806 (10 \mu M), a specific opener of I\text{to} [17] (Fig. 2I). The I\text{to} currents were elicited with clamp pulses at +40 mV from a holding potential of −80 mV. Compared with baseline (18.8 ± 0.85 pA/pF), peak current density significantly increased (24.9 ± 1.5 pA/pF; \(P < 0.05\); \(n = 6\)) after the addition of NS5806, but additional presence of NaHS (100 \mu M) returned the NS5806-stimulated currents to baseline levels (18.6 ± 0.6 pA/pF; \(P < 0.01\); \(n = 6\)) (Fig. 2J). After exposure to NS5806 (10 \mu M), inactivation of I\text{to} was significantly subdued, as reflected by an expansion in time constant (\(\tau\), from 8.6 ± 0.2 to 13.6 ± 0.7 ms at −30 mV, \(P < 0.05\); \(n = 6\)). However, addition of 100 \mu M NaHS returned the time constant to 10.1 ± 0.9 ms at −30 mV in the presence of 10 \mu M NS5806 (Fig. 2K), confirming inhibition of H₂S on I\text{to} current.

Besides BKCa and I\text{to} currents, an inward rectifier current activated by hyperpolarization voltage steps on a holding potential of −40 mV that was sensitive to Ba\textsuperscript{2+} (0.5 mM) was found, indicating the presence of NaHS (100 \mu M) reduced the peak current density of I\text{kr} (Fig. 2B). The inhibitory effects occurred within 1 min., reached saturation at 10 min. and could not be washed out (Fig. 3C). NaHS showed a voltage-dependent suppression of the I\text{kr} current on the I–V curve from −4.4 ± 0.1 pA/pF to −3.0 ± 0.1 pA/pF at −110 mV (\(P < 0.05\); \(n = 6\)) (Fig. 3D) and a dose-dependent inhibition of peak current density with an IC\text{50} of 78.9 \mu M (Fig. 3E).

A minority of the atrial fibroblasts (1%; 1/54 cells) were found to exhibit inward currents with 50 ms voltage steps between −60 and +70 mV from −80 mV holding potential in 10 mV increments that resembled sodium current, indicating that K+ currents represent the major ionic species in human atrial fibroblasts.

**H₂S inhibits proliferation of atrial fibroblasts via suppression of I\text{to} currents and gene expression.**

Inhibition of BKCa channel by paxilline, but not Na channel, has been reported to suppress proliferation of ventricular fibroblasts previously [18]. We investigated whether inhibition of the major K+ currents of BKCa and I\text{to} by H₂S similarly affected atrial fibroblast proliferation. Cell proliferation was found to be dose-dependently suppressed by paxilline (BKCa inhibitor), 4-AP (I\text{to} inhibitor) and Ba\textsuperscript{2+} (I\text{kr} inhibitor) (Fig. 4). Similarly, NaHS at 100, 300, 500 \mu M reduced cell proliferation by 33.1 ± 4.2%, 43.7 ± 3.1%, 58.4 ± 6.2%, respectively (\(*P < 0.05\); **P < 0.01 versus vehicle control; \(n = 10\)) without significant apoptotic effect observed at 300 \mu M (Fig. 4B). While naringenin (100 \mu M) had no effect on cellular proliferation, NS5806 (100 \mu M) enhanced fibroblast proliferation by 9.1 ± 5.0% (\(P < 0.05\); \(n = 10)\). However, NaHS (100 \mu M) reduced cellular proliferation by 29.1 ± 5.8% (\(P < 0.01\); \(n = 10)\) and 23.1 ± 4.8% (\(P < 0.05\); \(n = 10\)) in the presence of naringenin (100 \mu M) and NS5806 (100 \mu M), respectively, confirming additive inhibitory effects of H₂S on BKCa and I\text{to} currents in reducing cellular proliferation (Fig. 4C and D).

K\text{ATP} channel has been reported to affect cellular proliferation [19]. However, modulation of K\text{ATP} channel (30%; 22/73 cells) (Fig. 5A and B) and Kir6.2 (responsible for K\text{ATP}) gene expression (Fig. 5C) by H₂S while confirming its role in enhancing current density, failed to show any appreciable effect on proliferation of our atrial fibroblasts. The K\text{ATP} currents were elicited from voltage-clamped at

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**Fig. 3** Effect of NaHS on I\text{kr} currents in human atrial fibroblasts. (A) inwardly rectifying voltage-dependent currents were suppressed by Ba\textsuperscript{2+} (0.5 mM). Ba\textsuperscript{2+}-sensitive I–V relationships of the membrane currents of typical I\text{kr}. (B) I\text{kr} traces recorded in the absence and presence of NaHS (100 \mu M). (C) Time course of I\text{kr} current inhibition after addition of NaHS (100 \mu M). (D) Mean I–V relationship of peak I\text{kr} current in the absence and presence of NaHS (100 \mu M) (*P < 0.05 versus control). (E) A concentration response curve of NaHS-induced inhibition on I\text{kr} (*P < 0.05; **P < 0.01; \(n = 6\)).
Fig. 4 Effect of ion channel modulators on cell proliferation and apoptosis of human atrial fibroblasts. (A) Cell proliferation was assessed by BrdU assay in cells treated with Paxilline (0.3–3 μM), 4-AP (0.3–1 mM), Ba2+ (0.3–1 mM) or NaHS (100–500 μM) (*P < 0.05; **P < 0.01 versus basal levels; n = 10). (B) NaHS (1–300 μM) exerts no significant cellular apoptosis effect on cultured human atrial fibroblasts. (C) NaHS reverses fibroblast proliferation induced by Naringenin (Nari; BKCa opener, *P < 0.05 versus Nari alone). (D) NaHS suppresses cellular proliferation induced by NS5806 (Ito opener, *P < 0.05 versus basal levels; **P < 0.01 versus NS5806 alone; n = 10).

Fig. 5 Effect of NaHS on K<sub>ATP</sub> channels. (A) Superimposed K<sub>ATP</sub> current traces recorded in the absence and presence of NaHS (100 μM), pinacidil (30 μM) and glibenclamide (100 μM) (n = 6 in each group). (B) Graph representation of mean values of K<sub>ATP</sub> current in the absence and presence of NaHS (100 μM), pinacidil (30 μM) and glibenclamide (100 μM) (*P < 0.05; **P < 0.01 versus basal levels). (C) RT-PCR micrographs showing effect of 100 μM NaHS on Kir6.2 expression in atrial fibroblasts. Summary data displaying effect of NaHS on Kir6.2 expression. (**P < 0.01 versus basal levels; n = 4). (D and E) Cell proliferation was assessed in cells treated with glibenclamide (1–100 μM), pinacidil (1–100 μM) in the absence and presence of NaHS (100 μM). (*P < 0.05; **P < 0.01 versus basal levels; n = 4).
production of endogenous $H_2S$ by enhancing cystathionine $\gamma$-lyase (CSE) mRNA levels and maintaining its expression even in the presence of D,L-propargylglycine (PPG), a potent inhibitor of CSE (Fig. 6E and F). These results indicated that $H_2S$ inhibited fibroblast proliferation by regulating Kv4.3 mRNA expression and inhibiting $I_{to}$ current, possibly via an autocrine feedback mechanism.

**$H_2S$ inhibits TGF-β1–induced differentiation of atrial fibroblasts to myofibroblasts**

Transforming growth factor-β1 and Angiotensin II (Ang II) as the major mediators of fibroblast proliferation and their differentiation towards myofibroblasts in atrial fibrosis [20, 21] were consistently shown to promote proliferation of atrial fibroblasts in our study (Fig. 7A). Additional presence of NaHS decreased TGF-β1– (1 ng/ml), Ang II– (100 mM) and 20% FBS-induced fibroblast proliferation by 50.1 ± 4.3% (P < 0.01; n = 10), 42.1 ± 5.7% (P < 0.01; n = 10) and 21.2 ± 3.4% (P < 0.05; n = 10), respectively, which suggested $H_2S$ as a potent inhibitor of cytokine-mediated fibroblast proliferation. Furthermore, NaHS (100 μM) decreased TGF-β1–(1 ng/ml)–induced fibroblast transformation into myofibroblasts whereby mRNA expression of α-SMA, a hallmark of fibroblast differentiation, was significantly down-regulated (34.1 ± 7.1% reduction versus TGF-β1 alone; P < 0.05) (Fig. 7B), which was confirmed by reduced immunocytochemical α-SMA staining (percentage of α-SMA–positive cells, 47 ± 6% versus 90 ± 7%; P < 0.01; n = 4) (Fig. 7C and D). Nevertheless, no significant change in α-SMA–containing stress fibres was observed after NaHS treatment alone (percentage of α-SMA–positive cells, 33 ± 4%; n = 4) as compared with standard cultured atrial fibroblasts (32 ± 7%; n = 4) in 10% FBS.

**Discussion**

Multiple potassium channels are known to express in cardiac ventricular fibroblasts [15] and inhibition of BKCa current resulted in suppression of fibroblast proliferation [18]. Transient outward $K^+$ current, $I_{to}$, is present in neonatal rat cardiac fibroblasts (encoded by Kv1.4) [22] and human ventricular fibroblasts (encoded by Kv4.3) [15]. Similarly, $Ba^{2+}$-sensitive inward rectifier $K^+$ current (encoded by Kir2.1/ Kir2.3) is present in human ventricular fibroblasts [15] and rat ventricular fibroblasts [23] whereby its modulation may have major significance in cardiac fibrosis. However, their roles in atrial fibroblasts which are more actively participating in cardiac fibrosis [24], are relatively not well understood.

We demonstrated that $H_2S$ dose-dependently inhibited BKCa, $I_{to}$ and $I_{Kir}$ in human atrial fibroblasts within minutes, suggesting an acute modulation of $H_2S$ on such channels. The inhibitory effect of $H_2S$ on BKCa, $I_{to}$ and $I_{Kir}$ was observed at 25–400 μM. The physiological levels of plasma $H_2S$ have been reported to be 50–160 μM in human brain [25] and 50–100 μM in human serum [26]. As NaHS dissolved in saline, one-third of the $H_2S$ exists as an undissociated gas, and the remaining two-third as the HS⁻ anion [27]. Therefore,
the physiologically relevant concentration of H2S (25–400 μM) used in this study, which effectively blocked BKCa, Ito, and IKir in vitro, is likely to be attainable in vivo.

We found that NaHS attenuated naringenin-induced BKCa activation and decelerated the transition from closed to open state of the channel, suggesting a role for H2S in regulating BKCa channel kinetic and voltage sensitivity. However, NaHS had no effect on the half-maximum voltage activation, but shifted the steady-state inactivation curve to the left, indicating that the voltage-dependent steady-state inactivation kinetics of Ito channel were altered. Furthermore, NaHS markedly shifted the recovery curve of Ito to the right, indicating that H2S attenuated the recovery of Ito from inactivation. In contrast to reported presence of sodium current in ventricular fibroblasts (61%) [15], we found relatively few cells (1%) with detectable sodium current. This is consistent with previous reported presence of fast sodium current only in atrial myofibroblasts, but not in undifferentiated fibroblasts [28] like those used in our study.

BKCa channels (encoded by KCa1.1) have been demonstrated to regulate proliferation of human cardiac ventricular fibroblasts [18] and endothelial cells [29]. Furthermore, inhibition of IKir current suppressed proliferation of endothelial cells [30]. Similarly, inhibition of BKCa (by paxilline), Ito (by 4-AP) and IKir (by Ba2+) currents resulted in a significant reduction in fibroblast proliferation in our study. Consistently, suppression of the K+ currents by NaHS inhibited atrial fibroblast proliferation in a dose-dependent manner. Furthermore, suppression of proliferation by NaHS in the presence of naringenin (channel opener of BKCa) or NS5806 (channel opener of Ito) suggested an additive inhibitory effect of H2S on BKCa and Ito channels in proliferation of atrial fibroblasts. Consistently, H2S inhibition of lung fibroblast proliferation has been reported to be independent of KATP channel [32].

Consistent with electrophysiological findings on the presence of BKCa, Ito and IKir potassium currents, RT-PCR confirmed expression of KCa1.1, Kv4.3 and Kir2.1 in atrial fibroblasts. Furthermore, H2S decreased Kv4.3 expression and significantly moderated TGF-β1-mediated...
ated enhanced expression of Kv4.3 as well as KCa1.1 and Kir2.1. Effect of NaHS (exogenous donor of H₂S) on expression of cystathionine γ-lyase (CSE) that produces endogenous H₂S is controversial, with reports of no effect in human airway smooth muscle cells [33] to inhibitory effect in mouse aortic smooth muscle cells [34]. However, in concordance with other reports [27, 35], our results showed that NaHS enhanced CSE expression and further sustained its expression in the presence of DL-PPG[27] that strongly inhibited expression of CSE.

Myofibroblasts characterized by increased α-SMA expression are abundant in cardiac fibrosis [36] that has been associated with TGF-β-mediated [20] and Ang II-mediated [21] atrial fibrillation. Preventing myofibroblast differentiation from proliferating fibroblasts has been an attractive target in limiting cardiac fibrosis. Inhibition of TGF-β1 function by anti-TGF-β1 antibodies reduced myofibroblasts and lessened fibrosis [37]. Hydrogen sulphide was found to inhibit TGF-β-induced transformation of MRC5 lung fibroblasts to myofibroblasts [32]. Consistently, our results showed that NaHS effectively reduced proliferation of atrial fibroblasts in response to TGF-β1, Ang II or FBS. Furthermore, NaHS ameliorated transformation towards myofibroblasts whereby α-SMA expression and their stress fibres were significantly suppressed, although causal role of potassium channels in such transformation remained to be ascertained.

In summary, our study provides evidence of major K⁺ channels in human atrial fibroblasts that share similar heterogenous expression as in human ventricular fibroblasts [15]. Hydrogen sulphide inhibits fibroblast proliferation probably through a combined modulation of BKCa, I₉, 1KCa, but not K_ATP, channels. Although roles of MAPK and ERK pathways in our atrial fibroblasts remain to be determined, they were implicated in H₂S-mediated suppression of proliferation of vascular smooth muscle cells [38] and lung fibroblasts [32]. Both kinase pathways were linked to cell cycle progression in lung fibroblasts [39], which, in turn were reportedly regulated by BKCa in human ventricular fibroblasts [18]. However, K_ATP was found to play no significant role in ERK-inhibiting effect of H₂S [32], which may explain our observation in this study. Consistent with the observed beneficial effects of H₂S on cardiac fibrosis in vivo [12, 13], our results suggested that such effects may be partly mediated via selective inhibition of K⁺ channels in atrial fibroblasts and suppression of their transformation to myofibroblasts. Such regulating role of H₂S in atrial fibroblasts may have clinical value in targeting atrial fibrillation, which invariably linked to atrial fibrosis.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Immunocytochemical staining against anti-collagen I (top panel) and anti-human fibroblast antibodies in human atrial fibroblasts. Scale bar: 25 μm.

Figure S2 Immunocytochemical staining of Bkca (Kv1.1), Ito (Kv4.3) and IKir (Kir2.1) channels in human atrial fibroblasts. Scale bar: 50 μm.

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