Endogenous hydrogen sulfide contributes to uterine quiescence during pregnancy

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Abstract

Recent evidence suggests that uterine activation for labor is associated with inflammation within uterine tissues. Hydrogen sulfide (H₂S) plays a critical role in inflammatory responses in various tissues. Our previous study has shown that human myometrium produces H₂S via its generating enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS) during pregnancy. We therefore explored whether H₂S plays a role in the maintenance of uterine quiescence during pregnancy. Human myometrial biopsies were obtained from pregnant women at term. Uterine smooth muscle cells (UMSCs) isolated from myometrial tissues were treated with various reagents including H₂S. The protein expression of CSE, CBS and contraction-associated proteins (CAPs) including connexin 43, oxytocin receptor and prostaglandin F_{2α} receptor determined by Western blot. The levels of cytokines were measured by ELISA. The results showed that CSE and CBS expression inversely correlated to the levels of CAPs and activated NF-κB in pregnant myometrial tissues. H₂S inhibited the expression of CAPs, NF-κB activation and the production of interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNFα) in cultured UMSCs. IL-1β treatment reversed H₂S inhibition of CAPs. Knockdown of CSE and CBS prevented H₂S suppression of inflammation. H₂S modulation of inflammation is through K_{ATP} channels and phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways. H₂S activation of PI3K and ERK signaling is dependent on K_{ATP} channels. Our data suggest that H₂S suppresses the expression of CAPs via inhibition of inflammation in myometrium. Endogenous H₂S is one of the key factors in maintenance of uterine quiescence during pregnancy.

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Introduction

Preterm birth is the prevalent cause of mortality and morbidity in newborn infants. Moreover, the survivors of preterm birth have increased risk of neurodevelopmental impairments, gastrointestinal and respiratory complications (Liu *et al.* 2012). So far, it lacks effective methods of prediction or prevention for preterm birth. This failure is largely due to our incomplete understanding of the process that maintains uterus in a relatively noncontractile state throughout most of pregnancy and converts it into a procontractile state toward the end of pregnancy.

Human uterine conversion into the procontractile state results from the coordinated expression of a cassette of contraction-associated proteins (CAPs) such as gap junction, ion channels and the receptors of agonists (such as oxytocin and prostaglandins) (Challis *et al.* 2000), thereby making the uterus to respond to hormonal signals and mechanical forces.

In the past decade, increasing body of evidence suggests that uterine activation for labor in women is associated with inflammatory responses within uterine tissues (Romero *et al.* 2006, Rinaldi *et al.* 2011, Shynlova *et al.* 2013). Toward the end of pregnancy, uterine tissues display inflammatory response, which is characterized by an influx of inflammatory cells into the myometrium and cervix with concomitant increases in vascular and leukocyte adhesion molecule expression, activation of NF-κB and increased production of chemokines and proinflammatory cytokines in uterus (Thomson *et al.* 1999, Osman *et al.* 2003, Gomez-Lopez *et al.* 2011). However, the mechanisms underlying modulation of inflammatory response in uterus remain unclear.

Hydrogen sulfide (H₂S), the third endogenous gaseous transmitter, has been implicated to be involved in many physiological and pathophysiological processes including angiogenesis (Papapetropoulos *et al.* 2009), vasodilation (Skovgaard *et al.* 2011) and

inflammation (Wallace *et al.* 2012). H₂S is generated from L-cysteine principally through two pyridoxal-5-phosphate-dependent enzymes: cystathionine- γ -lyase (CSE, EC4.4.1.1) and cystathionine- β -synthetase (CBS, EC 4.2.1.22) (Li *et al.* 2011). Both CBS and CSE have been identified in gestational tissues including placenta and myometrium (Holwerda *et al.* 2012, Hu *et al.* 2016). Our previous study has shown that endogenous H₂S produced by CSE and CBS suppresses the spontaneous contractility of pregnant human myometrial strips (You *et al.* 2011). Furthermore, we have also demonstrated that CSE and CBS expression levels and production of H₂S in human myometrium are decreased with the onset of labor (You *et al.* 2011), suggesting that endogenous H₂S is involved in the initiation and progress of labor in women.

Based on the above background, we hypothesized that endogenous H₂S produced locally may play a role in the maintenance of uterine quiescence via modulation of uterine inflammation during human pregnancy. To test it, we firstly analyzed the correlation between the expression of CAPs and level of CSE and CSE in myometrium obtained from pregnant women at term. We then studied the effect of H₂S on the expression of CAPs, investigated whether H₂S regulates CAPs via the modulation of inflammatory responses and elucidated molecular mechanisms involved in a model of primary human uterine smooth muscle cells (USMCs). Our data indicate that endogenous H₂S contributes to the maintenance of uterine quiescence via inhibition of inflammation during pregnancy.

Materials and methods

Tissue collection

This study was approved by the specialty committee on ethics of biomedicine research, Second Military Medical University, Shanghai, China. Written informed consent was obtained from all patients. Biopsies of human myometrium were obtained at cesarean section from the following group of pregnant women: term no labor (TNL, $n=23$) and term labor (TL, $n=21$). The average gestational age of these two groups was 38 weeks, with a range of 37–42 weeks. Labor was defined as regular contractions (<5 min apart) plus membrane rupture and cervical dilation (>3 cm) with no augmentation (oxytocin or prostaglandin administration). None of the women included in this study had evidence of underlying disease (e.g. hypertension, diabetes, preeclampsia, intrauterine growth restriction, etc.). Biopsies were excised from the middle portion of upper edge of the incision line in the lower uterine segment. All myometrial samples were dissected free of serosa and immediately placed in Krebs' solution and maintained at 4°C and transported to the laboratory for cell culture or frozen immediately in liquid nitrogen and stored at -80°C for the subsequent Western blot analysis.

Cultures of USMCs

USMCs were isolated from TNL myometrial tissues as described previously (You *et al.* 2012). Briefly, myometrial tissue pieces were incubated with DMEM containing 1 mg/mL collagenase type II (Invitrogen) and 1 mg/mL deoxyribonuclease I (Sigma-Aldrich) at 37°C with shaking for 30 min for two times. After filtration, the cell suspension was centrifuged and the cell pellet was resuspended in DMEM containing 10% fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 mg/mL). The cells were then plated into 25 cm² flasks and kept at 37°C in 5% CO₂–95% air-humidified atmosphere until confluent (~2 week). The experiments were performed with the cells at passage 2. The cells were seeded in 12-well plates and then treated with the following reagents for 24 h: NaHS (5–100 $\times 10^{-6}$ M), L-cysteine (1.25–10 $\times 10^{-4}$ M), glibenclamide (10⁻⁵ M), PD98059 (10⁻⁵ M), LY294002 (10⁻⁵ M) and interleukin (IL)-1 β (1 ng/mL). The vehicle control was set without additive. In some cases, the cells were treated with NaHS (10⁻⁴ M), L-cysteine (10⁻³ M) or vehicle for 5, 10, 30 and 60 min. All of the previously mentioned reagents were purchased from Sigma-Aldrich. Each treatment was performed in triplicate for each preparation of cells. The concentrations of previously mentioned reagents were determined based on our previous studies (You *et al.* 2011, Hu *et al.* 2016) and preliminary data. To assess the purity of myocyte cultures, we routinely performed immunocytochemistry using α -actin monoclonal antibody (Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6, IL-1 β and tumor necrosis factor (TNF) α in culture media of USMCs were determined with specific ELISA (R&D Systems) according to the manufacturer's instructions.

Western blot analysis

Myometrial tissues were homogenized in cold T-Per lysis buffer (Pierce), and primary HUSMCs were harvested in the presence of M-Per lysis buffer (Pierce). The amounts of up to 50 μ g of protein samples were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After incubation with blocking buffer, the membranes were incubated with specific antibodies: CBS (ab131155, Abcam), CSE (ab133212, Abcam), connexin 43 (CX43) (ab11370, Abcam), oxytocin receptor (OTR) (sc-33209, Santa Cruz), prostaglandin F_{2 α} receptor (PGFR) (sc-67029, Santa Cruz), p65 (ab131485, Abcam), phospho-p65 (ser-529) (ab97726, Abcam), extracellular signal-regulated kinase (ERK)1/2 (4695, Cell signaling), phospho-ERK1/2 (4370, Cell signaling), phosphoinositide 3-kinase (PI3K) (sc-377482, Santa Cruz) and phospho-PI3K (Tyr508) (sc-12929, Santa Cruz) overnight at 4°C at a dilution of 1:1000. Then, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Santa Cruz). The chemiluminescent signal from the membranes was quantified by a GeneGnome HR scanner using GeneTools software (SynGene). To control

Table 1 List of primers used for the amplification of chemokines and cytokines in USMCs.

Name	Sense (S) and antisense (A) primer sequence (5'–3')	Genbank/EM BL accession no.	Production size (bp)
IL-1 β	A: 5'-GTGGCAATGAGGATGACTT-3' S: 5'-TGGGCTTATCATCTTTCAA-3'	NM_000576	537
IL-6	A: 5'-CCTTCCAAAGATGGCTGAAA-3' S: 5'-AGCTCTGGCTTGTTCCTCAC-3'	NM_000600	165
TNF- α	A: 5'-GCCCCAGAGGGAAGAGTTCCTCA-3' S: 5'-GCTTGAGGGTTGCTACAACATGGGC-3'	NM_000594	124

sampling errors, the ratio of band intensities to the β -actin was obtained to quantify the relative protein expression level.

Total RNA extraction and quantitative real-time RT-PCR

Total RNA was prepared from myometrial tissues and cells using TRIzol reagent (Invitrogen). Two micrograms RNA were reverse transcribed with oligo(dT)18 primer using the M-MLV reverse transcriptase (Promega). Specific primers for the amplification of IL-1 β , IL-6 and TNF α were listed in Table 1. Quantitative real-time PCR was carried out using Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The reaction solution consisted of 2.0 μ L diluted cDNA product, 0.2 μ mol/L of each paired primer, 200 μ mol/L deoxynucleotide triphosphates, 1 U Taq DNA polymerase (Qiagen) and 1 $\times$ PCR buffer. SYBRGreen (Roche) was used as a detection dye. The annealing temperature was set at 60°C and amplification cycles were set at 40 cycles. The temperature range to detect the melting temperature of the PCR product was set from 60°C to 95°C. To determine the relative quantitation of gene expression for both target and housekeeping genes, the comparative Ct (threshold cycle) method with arithmetic formulae was used (Li *et al.* 2016). Two reference genes β -actin and GAPDH were measured for each sample as an internal control for sample loading and normalization. Messenger RNA levels were normalized relative to β -actin and GAPDH values respectively. Because very similar results were obtained using these two reference genes, the results were illustrated using β -actin as internal control.

RNA interferences

The small interfering RNA (siRNA) for CBS and CSE were designed and synthesized by GenePharma Corporation (Shanghai, China). Control siRNA was scrambled sequence without any specific known target. The sequences for targeting human CSE and CBS are CSE sense: 5'-GGCCUUUGCUUCAGGUUUATT-3', antisense: 5'-UAAACCUGAAGCAAAGGCCTT-3'; CBS sense: 5'-CGGAACUACAUGACCAAGUTT-3', antisense: 5'-ACUUGGUCAUGUAGUCCGTT-3'. Transfection of siRNA was performed by using Lipofectamine TM 2000 as described previously (You *et al.* 2011, Hu *et al.* 2016).

Statistical analysis

Data are presented as mean $\pm$ S.E.M. In some cases, for illustrative purposes, the results are presented as the mean percent control $\pm$ S.E.M. All data were tested for homogeneity of variance by Bartlett's test before statistical analysis. The data

of CAP expression and NF κ B activation in TNL and TL tissues were analyzed by *t* test. Pearson's correlation was used to explore the relationships of CBS or CSE with CX43, OTR, PGFR and activated NF- κ B level in myometrium. The data of cultured USMCs were analyzed by a one-way ANOVA followed by a Student–Newman–Keuls test. A *P* value <0.05 was considered significant.

Figure 1 The protein levels of CBS, CSE, p-p65, CX43, OTR and PGFR, in pregnant myometrium before and after onset of labor. Myometrial tissues were obtained from pregnant women at term before (TNL) or after the onset of labor (TL). Semi-quantitation of Western blot signals of CBS (A), CSE (B), p-p65 (C), CX43 (D), OTR (E) and PGFR (F) in TNL and TL myometrium biopsies. Representative protein bands were shown on the top of each histogram. Data were presented as mean $\pm$ S.E.M. **P < 0.01 vs TNL.

Results

The expression levels of CSE and CBS correlate to levels of CAPs and NF- κ B in human pregnant myometrium

As expected, the protein levels of CSE and CBS were downregulated in TL myometrial tissues compared with TNL tissues (Fig. 1A and B). Prior studies have shown that the expression of CAPs, such as CX43, OTR and PGFR, and the level of phospho-p65 (p-p65), active form of NF- κ B are increased with labor (Condon *et al.* 2006, Kamel 2010). We therefore examined the protein levels of these proteins and found that the levels of CX43, OTR, PGFR and phospho-p65 were significantly increased in TL myometrial tissues compared with TNL tissues (Fig. 1C, D, E and F). The correlation analysis showed that the level of CSE and CBS inversely correlated to CX43, OTR, PGFR and active NF- κ B level (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

NaHS and L-cysteine inhibit the expression of CX43, OTR and PGFR in USMCs

We then investigated whether H₂S modulates CX43, OTR and PGFR expression in primary USMCs. As shown in Fig. 2A, B and C, treatment of USMCs with increasing concentration of NaHS (5×10^{-6} – 10^{-4} M), the H₂S donor, caused a decrease in the expression of CX43, OTR and PGFR in a dose-dependent manner in a 24-h incubation time. L-cysteine (1.25 – 10×10^{-4} M), the precursor of H₂S, treatment also dose-dependently

suppressed CX43, OTR and PGFR expression (Fig. 2D, E and F).

NaHS and L-cysteine suppresses NF κ B activation and production of proinflammatory cytokines in USMCs

Prior studies have shown that H₂S suppresses NF- κ B activation in macrophages (Du *et al.* 2014). We therefore examined whether H₂S treatment affects NF- κ B activation in USMCs. As shown in Fig. 3A and B, treatment of cells with NaHS (10^{-4} M) and L-cysteine (10^{-3} M) for 5–60 min decreased p-p65 level in a time-dependent manner.

As NF- κ B plays a key role in driving cytokine and chemokine production. We explored whether H₂S modulates the production of proinflammatory cytokines and chemokines. As shown in Fig. 3C, D and E, NaHS (5×10^{-6} – 10^{-4} M) treatment dose-dependently inhibited IL-1 β , IL-6 and TNF α output in USMCs. Treatment of the cells with L-cysteine also caused a decrease in the output of IL-1 β , IL-6 and TNF α in a dose-dependent manner (Fig. 3F, G and H). Both NaHS and L-cysteine significantly inhibited IL-1 β , IL-6 and TNF α mRNA expression (Fig. 3C, D, E, F, G and H). However, NaHS and L-cysteine had no effect on the output of chemokines CCL-2 and CXCL-8 (Supplementary Fig. 2).

The role of CSE and CBS in L-cysteine suppression of proinflammatory cytokine production in USMCs

To explore the role of CSE and CBS in the effects of L-cysteine on inflammatory cytokine production, small interfering RNA (siRNA) approach targeting CSE and

Figure 2 The effects of hydrogen sulfide on the expression of CX43, OTR and PGFR in USMCs. USMCs were treated with increasing concentration of NaHS (5×10^{-6} – 10^{-4} M) or L-cysteine (1.25 – 10×10^{-4} M) for 24 h. The cells were then collected and protein levels of CX43 (A and D), OTR (B and E) and PGFR (C and F) were determined by Western blot. Values are presented as mean $\pm$ S.E.M. for $n=6$ subjects performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs vehicle control. L-cys, L-cysteine.

Figure 3 The effects of hydrogen sulfide on NF-κB activation and the production of cytokines in USMCs. (A, B, C, D, E, F, G and H) USMCs were treated with NaHS (5×10^{-6} – 10^{-4} M) or L-cysteine (1.25 – 10×10^{-4} M) for 24 h. The cells were collected for determination of the levels of protein levels of p-p65 (A and B) using Western blot and mRNA levels of IL-1β (C and F), IL-6 (D and G) and TNFα (E and H) by quantitative real-time RT-PCR. The supernatant was collected and levels of IL-1β (C and F), IL-6 (D and G) and TNFα (E and H) were measured by ELISA. Values are presented as mean $\pm$ S.E.M. for $n=6$ subjects performed in triplicate. Representative protein bands of p-p65 were on the top of each histogram. (I, J and K) USMCs were transfected with siRNA targeting CSE or CBS for 10 h and changed medium with DMEM for 14 h and then followed by L-cysteine (10^{-4} M) treatment for 24 h. The supernatant was collected and levels of IL-1β (I), IL-6 (J) and TNFα (K) were measured by ELISA. Values are presented as mean $\pm$ S.E.M. for $n=3$ subjects performed in triplicate. * $P<0.05$, ** $P<0.01$ vs vehicle control.

CBS was applied. The protein level of CSE and CBS was decreased by about 68% and 91% respectively, upon transfection with CSE and CBS siRNA (Supplementary Fig. 3). As shown in Fig. 3G, H and I, knockdown of either CSE or CBS reversed L-cysteine inhibition of the output of proinflammatory cytokines.

IL-1β blocks NaHS and L-cysteine suppression of CX43, OTR and PGFR expression

We then investigated the effects of proinflammatory cytokines on H₂S inhibition of the expression of CAPs. As shown in Fig. 4, the inhibitory effect of H₂S on CX43,

Figure 4 The effects of H₂S on CX43, OTR and PGFR expression in the presence of IL-1β. USMCs were treated with NaHS (5×10^{-6} – 10^{-4} M) or L-cysteine (1.25 – 10×10^{-4} M) in the presence of IL-1β (1 ng/mL) for 24 h. Cells were collected for determination of the protein levels of CX43 (A and D), PGFR (B and E) and OTR (C and F) by Western blot analysis. Representative protein bands are presented on the top of the responding histogram. Values are presented as mean $\pm$ S.E.M. for $n=4$ subjects performed in triplicate. * $P<0.05$, ** $P<0.01$ vs vehicle control. L-cys, L-cysteine.

OTR and PGFR expression did not occur in the presence of IL-1 β .

The role of K_{ATP} channels in NaHS and L-cysteine suppression of proinflammatory cytokines

We explored the role of K_{ATP} channels in H₂S suppression of proinflammatory cytokines because K_{ATP} channels have been reported to mediate H₂S action in many studies (Jiang *et al.* 2010, Tang *et al.* 2010). As shown in Fig. 5A, B and C, K_{ATP} channel inhibitor glibenclamide (10⁻⁵ M) reversed NaHS and L-cysteine inhibition of IL-1 β , IL-6 and TNF α output.

PI3K and ERK signaling pathways are involved in NaHS and L-cysteine suppression of proinflammatory cytokines

Prior studies have also reported that H₂S can activate PI3K and ERK signaling in some tissues (Hu *et al.* 2008, Tamizhselvi *et al.* 2009, Ang *et al.* 2011, Peake *et al.* 2013). We therefore examined the role of PI3K and ERK signaling in the H₂S modulation of proinflammatory cytokine output. It was found that the PI3K inhibitor LY294002 and ERK inhibitor PD98059 could block the inhibitory effects of NaHS and L-cysteine on IL-1 β , IL-6 and TNF α production (Fig. 5D, E and F).

As shown in Fig. 5G, H, I and J, treatment of cells with NaHS (10⁻⁴ M) and L-cysteine (10⁻³ M) for 5–60 min increased phospho-PI3K (p-PI3K) and phospho-ERK1/2 (p-ERK1/2) levels in a time-dependent manner.

NaHS and L-cysteine activation of PI3K and ERK signaling is dependent on K_{ATP} channels

We investigated whether H₂S activation of PI3K and ERK signaling is associated with activation of K_{ATP} channels. As shown in Fig. 6A and B, in the presence of glibenclamide (10⁻⁵ M), the effects of NaHS and L-cysteine treatment on PI3K and ERK activation did not occur.

We then examined whether H₂S suppression of NF- κ B activation is dependent on K_{ATP} channels. Glibenclamide (10⁻⁵ M) treatment did not affect NaHS and L-cysteine inhibition of p-p65 level (Fig. 6C and D).

Discussion

The present study showed for the first time that both H₂S donor NaHS and H₂S precursor L-cysteine exhibited suppressive effects on CAP expression and NF- κ B activation in cultured USMCs obtained for pregnant myometrial biopsies. NaHS and L-cysteine also inhibited the production of proinflammatory cytokines including IL-1 β , IL-6 and TNF α . Knockdown of CSE or CBS reversed suppressive effects of L-cysteine on inflammation, confirming the effects of endogenous H₂S produced via CSE and CBS. Moreover, IL-1 β blocked the inhibitory effect of H₂S on the expression of CAPs in myometrium. Thus, our study indicated that H₂S suppresses uterine activation by inhibition of inflammation.

Many previous studies have reported that H₂S has anti-inflammatory actions in various tissues, such as inhibition of leukocyte adhesion and the release

Figure 5 The roles of K_{ATP} channels, ERK and PI3K signaling pathways in the effect of H₂S on proinflammatory cytokine production. (A, B, C, D, E and F) USMCs were treated with NaHS (10⁻⁴ M) or L-cysteine (10⁻³ M) in combination with K_{ATP} channel inhibitor glibenclamide (10⁻⁵ M), ERK inhibitor PD98059 (10⁻⁵ M) and PI3K inhibitor LY294002 (10⁻⁵ M) for 24 h. The supernatant was collected and levels of IL-1 β (A and D), IL-6 (B and E) and TNF α (C and F) were measured by ELISA. (G, H, I and J) USMCs were treated with NaHS (10⁻⁴ M), L-cysteine (10⁻³ M) or vehicle for indicated time. Cells were collected and Western blot analysis was used to determine protein levels of ERK1/2 (G and H), p-ERK1/2 (G and H), PI3K (I and J) and p-PI3K (I and J). Representative protein bands are presented on the top of the responding histogram. Values are presented as mean $\pm$ S.E.M. for $n=4$ subjects performed in triplicate. * $P<0.05$, ** $P<0.01$ vs vehicle control. Gliben, glibenclamide.

Figure 6 The role of K_{ATP} channels in H₂S activation of PI3K and ERK signaling and suppression of NF-κB activation. USMCs were treated with NaHS (10⁻⁴ M) or L-cysteine (10⁻³ M) in the presence of glibenclamide (10⁻⁵ M) for indicated time. Cells were collected and Western blot analysis was used to determine protein levels of ERK1/2 (A and B), p-ERK1/2 (A and B), PI3K (C and D), p-PI3K (C and D), p-65 (E and F) and p-p65 (E and F). Representative protein bands are presented on the top of the responding histogram. Values are presented as mean ± S.E.M. for *n* = 4 subjects performed in triplicate. *P < 0.05, **P < 0.01 vs vehicle control.

of inflammatory mediators (Tamizhselvi *et al.* 2009, Ang *et al.* 2011, Du *et al.* 2014, Li *et al.* 2016). However, some studies also demonstrated that H₂S could act as a proinflammatory mediator in the inflammatory responses (Bhatia *et al.* 2005, 2008, Ang *et al.* 2011). More recently, Badiei and coworkers (Badiei *et al.* 2016) reported that knockdown of CSE in these cells can protect the mice against pancreatitis, and therefore, proposed that endogenous H₂S produced by CSE is an endogenous inflammatory mediator in monocytes/macrophages. The present study showed that exogenous H₂S and H₂S produced by CSE and CBS suppressed NF-κB activation and inhibited IL-1β, IL-6 and TNFα production in USMCs. Collectively, it may suggest that

the effect of H₂S on inflammatory response is dependent on the machinery of the target tissues.

Some studies have demonstrated that H₂S affects the expression of proinflammatory cytokines by modulating NF-κB activity (Guo *et al.* 2013, Du *et al.* 2014). In consistence with these studies, we also found that H₂S suppressed the level of phospho-p65, the active form of NF-κB, in myometrial biopsies. Of note, K_{ATP} channels were firstly identified to mediate the dilatory effects of H₂S in vasculatures (Tang *et al.* 2010). Our previous studies have shown that H₂S suppresses the spontaneous contraction of human myometrial strips via K_{ATP} channels (You *et al.* 2011). In the present study, we demonstrated that blockage of K_{ATP} channels reversed H₂S suppression of proinflammatory cytokines. In addition, we showed that the impact of H₂S on NF-κB activity was not dependent on K_{ATP} channels. A number of studies have implicated that H₂S actually exerts its function by modifying L-cysteine in a large number of proteins by S-sulfhydration (Mustafa *et al.* 2011, Du *et al.* 2014, Módos *et al.* 2016, Sun *et al.* 2016). Moreover, it has been reported that H₂S regulates NF-κB activity and K_{ATP} channels via sulfhydration of L-cysteine in these proteins (Mustafa *et al.* 2011, Du *et al.* 2014). Taken together, it suggests that H₂S suppresses the production of proinflammatory cytokines by regulating the activity of NF-κB and K_{ATP} channels in pregnant USMCs.

The present study also showed that PI3K and ERK signaling pathways were involved in H₂S suppression of inflammation. Many studies have demonstrated that PI3K signaling is a negative regulator during inflammatory responses, such as lipopolysaccharide (LPS)-induced inflammatory response (Schabbauer *et al.* 2004, Luyendyk *et al.* 2007, Zhang *et al.* 2007, Bi *et al.* 2016). For instance, Zhang and coworkers (Zhang *et al.* 2007) reported that α-lipoic acid attenuates LPS-induced inflammation via activation of PI3K signaling. Moreover, in pancreatic cells, it has been shown that H₂S suppresses caerulein-induced IL-1β and TNFα production via PI3K signaling (Bi *et al.* 2016). Many studies have demonstrated that ERK signaling also promotes inflammatory responses in many tissues (Guha & Mackman 2001, Maeng *et al.* 2006, Mandrekar & Szabo 2009, Youn *et al.* 2016). However, some other studies reported that ERK signaling can serve as an anti-inflammation signaling in some tissues. Maeng and coworkers (Maeng *et al.* 2006) showed that activation of ERK suppresses NF-κB-dependent genes in endothelial cells. More recently, Subedi and coworkers (Subedi *et al.* 2016) demonstrated that the extract of *Lindera neesiana*, an herb medicine, decreased the production of proinflammatory cytokines and nitric oxide via ERK signaling pathway in neural cell lines. With regard to the mechanism by which H₂S induces PI3K and ERK signaling pathways, we found that H₂S activation of PI3K and ERK signaling is dependent on K_{ATP} channels, which is consistent with the study by Hu

and coworkers (Hu *et al.* 2008) in which they showed that H₂S activates PI3K and ERK signaling via K_{ATP} channels in cardiomyocytes.

Human parturition is an inflammatory process, which includes increased the expression and secretion of inflammatory cytokines and chemokines in uterus. Our previous study has demonstrated that human parturition is associated with the activation of inflammatory processes in uterus as evidenced by the activation of NFκB leading to increased expression of CAPs in myometrium (You *et al.* 2014). In the present study, we showed that downregulation of H₂S-producing enzymes CSE and CBS is associated with increased expression of CAPs and level of active NF-κB in myometrium during pregnancy. *In vitro* study, we found that H₂S suppression of CAP expression, NF-κB activation and the production of proinflammatory cytokines. Collectively, our data show that endogenous H₂S produced locally play a critical role in the maintenance of uterine quiescence, and this effect is associated with suppression of inflammation in uterus.

In conclusion, H₂S produced by CSE and CBS suppresses the expression of CAPs via inhibition of NF-κB and proinflammatory cytokines in pregnant human myometrial cells. The inhibitory effect of H₂S on proinflammatory cytokines is dependent on activation of K_{ATP} channels. Our data indicate that H₂S plays a critical role in the maintenance of uterine quiescence during pregnancy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0549>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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